A METHOD OF INCREASING PRECISION IN VITAMIN A ASSAY[1]

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The difficulties encountered in assaying foods and pharmaceutical materials for vitamin A are not adequately met, in the opinion of many workers, by the directions of the committee for revision of the U. S. Pharmacopoeia ('34). Richards ('35), for example, has presented a severe criticism of the plan of the test. In general practice too often, disconcerting variation has been the rule. The many rats failing to survive the long experimental period also has complicated results. While the recently recommended procedure of feeding carotene to one lot of an assay series has improved the reliability of the test, it involves the hazard of the unexplainable variations so often encountered in the response of the animals in the reference group itself.

Success in assay work has been attained in our laboratory only with the use of large experimental lots. With them, we have been able to obtain graded response at graded levels of feeding and to reproduce results at a specific dosage in consecutive years. Since 1933 we have been investigating the possibilities of increasing the accuracy of the assay by two methods: the shortening of the assay period, and the elimination of animals showing indications in the depletion period of erratic response to the test. The results have been fairly

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satisfactory. This report deals with the results of these experiments.

EXPERIMENTAL

During the fall, winter and spring months of 1932–1935, 557 standard test animals depleted of their bodily stores of vitamin A were fed quantitative graded supplements of carotene, canned tomatoes, or sweet potatoes, the level of dosage ranging from approximately 1.0 to 3.0 Sherman units daily. The carotene used was the provisional standard material first supplied by the League of Nations ('31).

The animals were of Wistar stock, strain A, inbred by brother and sister matings for sixty-five generations from representatives of the second litter. Average increments in weight at successive age intervals in these rats were the same from generation to generation and in composite samples of mixed generations from year to year (Timson, '32). Thus, a uniform average rate of growth appears to have been fixed by inbreeding.

Female rats only of our particular strain of animals are suitable for vitamin A assay work. Of the 557 females depleted of their bodily stores of vitamin A, 91% survived an experimental period of 8 weeks whereas only 58% of a group of 469 brother males lived throughout this period.

The test animals were taken from litters reduced to eight rats during the first week of life. When given the A-free ration, their mean weight was 43.5 gm., with standard deviation, 14.5 gm.

The constituents of the stock diet [2] were kept as constant as possible in regard to vitamin A value from season to season. A supply of whole corn, sufficient to meet the needs of the

[2] The percentage composition of the basal stock diet was as follows: ground yellow corn, 64; linseed meal, 16; crude casein, 5; alfalfa, 2; NaCl, 0.5; $CaCO_3$, 0.5; yeast, 1.5; irradiated yeast, 0.5; wheat germ, 10. This diet was supplemented daily with liquefied Klim to which was added cod liver oil (1 teaspoon per quart) and trace minerals (2 cc. per quart of a solution containing KI, $MgSO_4$, $K_2Al_2(SO_4)$, and $CuSO_4$). In addition, the rats received 5 gm. of raw lean beef and 10 gm. of lettuce on alternate days.

laboratory for a 4-year period was purchased and stored, small quantities being ground as needed. Once every year, a supply of dried winter milk [3] representing 1 day's run in the factory was purchased. Recorded data [4] show that these procedures measurably reduced variability in the time required for exhaustion of bodily stores of vitamin A, especially from season to season.

The animals were housed individually in cages with raised wire bottoms. Care was exercised in maintaining the cleanliness of the cages, particularly of the water cups (Ewe, '32).

The basal ration contained the following ingredients: casein (vitamin A-free,[5] 18%; hydrogenated lard,[6] 22%; cornstarch, 56%; Osborne and Mendel salt mixture,[7] 4%; 0.5 gm. of yeast,[8] one-fifth of which was irradiated.[9] The yeast was fed separately to each rat daily, thus insuring an adequate level of the B-complex in the diet after the rat voluntarily decreased its food intake.

Five-tenths gram of yeast per rat per day (approximately 8% of the diet) supplementing an otherwise adequate diet was shown to promote normal growth during the period of the experiment.[10] The yeast contained no traces of vitamin A (Honeywell, Dutcher and Ely, '31), no lengthening of the depletion period being observed when the level of yeast was increased to 40%. The irradiation of part of the yeast furnished adequate vitamin D as shown by appropriate assays.[11] More uniform depletion records were obtained with

[3] Klim, The Borden Co., New York.

[4] Unpublished data, nutrition laboratory of the Foods and Nutrition Department, Iowa State College.

[5] Crude casein, Wilkens-Anderson Company, Chicago, extracted four times (each extraction period, 1 hour long) with hot 95% alcohol, washed thoroughly between each extraction, and finally air-dried.

[6] Clix, Cudahy Packing Co., Chicago.

[7] J. Biol. Chem., vol. 32, p. 309, 1917.

[8] Yeast Foam Tablet Powder, the Northwestern Yeast Co., Chicago.

[9] Two hundred and fifty grams of yeast spread over an area measuring 12 × 16 inches was irradiated for ½ hour, with frequent stirring, 18 inches from arc of a quartz mercury vapor lamp.

[10] See footnote 4.

[11] We are indebted to Dr. B. H. Thomas of the animal chemistry nutrition subsection of the Iowa Agricultural Experiment Station, and to Dr. J. F. Edwards of the department of hygiene for these determinations.

the use of irradiated yeast than when D was supplied either by the irradiation of the animal or of the basal diet.[12]

Clix was used as the dietary fat because smaller increments of growth were observed in the depletion period with this fat in the ration than when Crisco was employed.[12] The use of fat in the diet prevented xerophthalmic symptoms due to irritation from food dust (Bauman and Steenbock, '34).

A normal rate of growth was induced by the addition of only 1.5 mg. of a standard cod liver oil to the A-deficient diet, evidence, we believe, that the feeding of the A-free ration produced an uncomplicated dietary deficiency inasmuch as the diet had already been proved adequate in vitamin D. None of the difficulties reported by Coward, Key and Morgan ('29) in a similar supplementation were experienced.

The rats were depleted of their bodily stores of vitamin A in 22.6 days (standard deviation, 10.0) which is approximately the time (23.6 days) that persistence of cornified cells in their vaginal smears was observed. Only the mere beginnings of xerophthalmia were present.

In order to meet Richards' criticism ('35), we gassed a group of animals that we considered adequately depleted and examined them for pathological lesions. A slight hyperemic condition of the intestinal capillaries and a trace of blood in the feces were the only abnormalities found. After 28 days, advanced xerophthalmia, chalky teeth, hemorrhage of intestinal capillaries and pus pockets in the ears and nose were noted in the animals examined.

Criteria for judging the exhaustion of vitamin A reserves were developed in this laboratory after 2 years of critical study because indices described in the literature (Sherman and Munsell, '25; Holmes, '32; U. S. Pharmacopoeia Committee of Revision, '34) were found somewhat too severe to apply in our particular experimental situation. These were: 1) maintenance of constant weight [13] for a period of 5 days

[12] See footnote 4, page 105.

[13] Constant weight is defined as that weight that does not alter more than ± 4 gm. from day to day. Such leeway in definition considers the effect of diurnal variation.

and incipient xerophthalmia;[14] 2) maintenance of constant weight for 4 days and slightly advanced xerophthalmia;[14] 3) severe xerophthalmia[14] only; and 4) a drop in body weight of 4 or more grams sustained for 2 days. If any one condition prevailed, the animal was considered depleted. The interval from the time that the animal first began to show signs of vitamin exhaustion to the time when it was ready for supplementary feeding was called 'the critical period.' Accuracy of judgment was greatly improved when the practice of weighing and observing of the animals daily throughout the depletion period was initiated.

In the preparation of the test lots every effort was made to obtain representative random samples. At depletion, the individuals of a litter were distributed insofar as possible in the various lots representing an assay. A sufficient number of breeding animals was used to insure the placing of approximately fifteen animals in each lot within a period of 30 days. Complete records were kept in both the depletion and growth periods of the animals.

RESULTS ATTAINED WITH METHOD DESCRIBED

Since an earlier report from this laboratory (Irwin, Brandt and Nelson, '30), the most significant variations in routine procedure introduced were: change in indices for determination of depletion of bodily stores of vitamin A, offering of yeast separate from the basal ration, a tested dietary source of vitamin D, the use of females only in the test, and a standardization of the stock colony ration. The standard deviation from the mean gain (32 gm.) of a group of 349 animals fed sources of vitamin A for 8 weeks as reported by Irwin, Brandt and Nelson was 53 gm., in the 557 rats used in assay work in

[14] A definite progression may be noted in the development of xerophthalmia (Steenbock and Wirick, '31). We recognize 3° of xerophthalmia in judging depletion, i.e., inicpient xerophthalmia, characterized by a slight swelling of the eyelids or a small exudate in the corner of the eye; slightly advanced xerophthalmia in which an exudate extends halfway around a reddened eye; severe xerophthalmia in which the entire eye is surrounded by exudate and the lids are very swollen and inflamed.

the laboratory since then, the standard deviation was 22 gm. (mean gain, 35 gm.). Thus, the details of standardization described in the previous section were effective in improving the assay.

In table 1 is a summary of results from forty-nine tests involving 439 rats. The tests are grouped in ten lots according to the level and supplement fed. Analysis of variance of each lot (Fisher, '30) showed that the test differences were in no case significant. The ten lots, therefore, were each assumed to be homogeneous for gain. The 138 rats whose records are not included in table 1 fell into miscellaneous tests, many representing only one assay.

Coward, in 1932, found the standard deviations from the mean gains were fairly uniform over a large number of test lots and furthermore, that the standard deviation was independent of the mean increase in weight of the test group. We thereupon calculated the standard deviations of the gains made by the ten lots of rats during the 8-week test period. Although these groups had been given different levels of vitamin A, considerable similarity in the results was observed. All but one of the ten calculations fell within the range, 17 to 20 gm. (table 5). Thus, we felt justified in assuming that the average of the ten standard deviations represented a measure of the variation in gains that might occur in any test group under the conditions of our test. This average standard deviation was found to be 18.1 gm.[15] The variation is essentially that demonstrated in the growth of stock rats of the same age in the same interval of life history (Timson, '32).

THE ELIMINATION OF ANIMALS ESTIMATED AS TOO VARIABLE FOR ASSAY

In our routine assay work, on the basis of a standard deviation of 18.1 gm., we were forced to use large groups of animals and our assays became exceedingly laborious, time-consuming,

[15] The average standard deviation was obtained by the formula $\sqrt{\frac{sd^2}{n}}$ in which sd^2 represents the pooled sum of the squares of the mean differences obtained for each of the ten lots and n represents the degrees of freedom within the lots.

TABLE 1

Summary of forty-nine tests involving 439 rats

LOT NO.	NUMBER IN LOT	SUPPLEMENT FED	MILLIGRAMS OF SUPPLEMENT	AVERAGE GAINS IN GRAMS IN DIFFERENT TEST PERIODS										VALUES OF z [1]	
				1	2	3	4	5	6	7	8	9	10	From the lot	At the 5% level
I	108	Sweet potato	35	39.9	44.4	48.5	50.8	37.4	45.5	41.9	60.7	51.6	45.0	0.3905	0.4090
II	40	Sweet potato	30	40.3	48.7	46.0	52.0	43.5						0.4046	0.4947
III	39	Sweet potato	25	28.2	53.6	30.2	42.7	38.9						0.6431	0.8767
IV	17	Sweet potato	20	13.2	50.2	33.2	46.7	50.2						0.7423	0.8767
V	27	Tomato	175	54.6	40.5	58.2	49.7							0.3128	0.5427
VI	69	Tomato	125	46.8	37.5	28.0	41.0	40.7	46.8	40.1	42.1	37.5		0.3415	0.3702
VII	56	Tomato	100	20.6	28.5	32.2	35.0	28.5						0.0899	0.4947
VIII	22	Carotene	0.001	61.1	47.2									0.5416	0.7386
IX	36	Carotene	0.0005	32.7	34.7									1.0821	2.7588
X	23	Carotene	0.0004	25.0	21.0									0.2920	0.7141

[1] Fisher, '30, section 41.

and expensive. We, therefore, next attacked the problem of reducing the average variability of the gains of our test rats. As all investigators know, no rejection of animals can be made on the basis of gross observations at the end of the depletion period. The only possible line of approach for our study seemed to lie in a determination of possible relationships that might exist between data collected in the depletion and assay periods of the test.

The laboratory records permitted the tabulation of the following items descriptive of the animal in the depletion period: age at weaning, weight at weaning, weight at end of depletion period, the number of days required for depletion, the total gain during depletion, the number of days constant weight was maintained in the critical period, the severity of the xerophthalmia, the gain or loss during the critical period, and the total quantity of food consumed during the depletion period. There were also complete records pertaining to the growth of the animals and the plane of food consumption in the assay period when some source of vitamin A was fed.

These data led us to ask two questions. First, what is the relationship between the quantity of food consumed and rate of growth when vitamin A is added to the deficient diet. Second, what is the relationship between data describing the behavior of the animal in the interval of depletion and the later growth induced by vitamin feeding.

Simple correlation of the factors involved answered the first question, coefficients being determined for all lots of rats represented in this study. No significant relation was found to exist between food intake and concomitant growth, no matter what level of vitamin A was fed.

In regard to the second question, multiple regression showed that of the nine depletion-history factors listed above, six contributed important information about subsequent growth. These are shown in table 2 where all the standard partial regression coefficients (Wallace and Snedecor, '31) are presented. Three were of outstanding importance in determining later gain. For example, the intake of food during the de-

pletion period was directly related to later growth increments whereas the time required for depletion and rate at which the rat grew in this interval bore inverse relationships to the gain made in the actual assay period. Actually, body weight at the end of the depletion period, as shown by simple correlation,

TABLE 2

Multiple correlation between behavior in depletion period and later growth in assay period

Multiple correlation of nine factors [1] and gain in 8 weeks, 0.394
Multiple correlation of six factors [1] and gain in 8 weeks, 0.388
Multiple correlation of six factors [1] and gain in 5 weeks, 0.385

FACTORS	STANDARD PARTIAL REGRESSION COEFFICIENTS SHOWING RELATION OF VARIABLE TO GAIN			DOES VARIABLE CONTRIBUTE IMPORTANT INFORMATION ABOUT GAIN?
	Nine factors and 8-week test period	Six factors		
		8-week test period	5-week test period	
Age at weaning	— 0.0044	.....	.....	No
Weight at weaning	— 0.1308	— 0.1590	— 0.1723	Yes
Weight at end of depletion	— 0.0660	.....	.....	No
Number of days required for depletion of body stores of vitamin	— 0.3670	— 0.2949	— 0.3852	Yes
Total gain in weight during depletion period	— 0.2536	— 0.3471	— 0.1848	Yes
Critical period				
Number of days of maintenance of constant weight	— 0.1545	— 0.1675	— 0.0880	Yes
Severity of xerophthalmia	+ 0.0668	.....	.....	No
Gain in weight	+ 0.1183	+ 0.1263	— 0.0572	Yes
Weight of total food intake during depletion period	+ 0.3451	+ 0.3217	+ 0.2317	Yes

[1] Those factors contributing important information about gain in test period.

was an important factor but its influence was eliminated in the multiple regression because this weight was the sum of the original weight of the animal and the gain it made in the depletion period.

No information was lost when the items, unimportant insofar as later gain was concerned, were eliminated from the study because multiple correlation coefficients calculated on the basis of nine and six factors, respectively, were found

to be nearly identical, i.e., 0.394 and 0.388. In a group of this size, coefficients as large as these show a highly significant relationship between factors studied.

Since the growth-determining factors were all found in depletion period, expected gain in the assay period was now expressed in terms of a regression equation in which gain in body weight became the dependent variable and the six correlated factors, the independent variables. With the equation established, the response of any animal to vitamin A feeding could be estimated when the data pertaining to its depletion history were used in solving the equation.

The data allowed the calculation of ten different regression equations, each based on the response of a lot to a specific dosage of vitamin A. However, since simple correlation coefficients obtained in a series of preliminary studies showed that the relation between these depletion factors and subsequent growth, were of the same order in each lot, the same relationship could be expected to hold within limits, in a composite group fed various levels of the vitamin. The equation finally obtained based on data pertaining to 577 animals, read as follows:

$$\bar{X} = 107.24 - 0.38A - 1.74B - 0.54C - 3.46D + 1.16E + 0.18F$$

where A = weight in grams at weaning; B = number of days required for depletion of bodily stores of the vitamin; C = total gains in grams during depletion; D = number of days of constant weight at end of depletion period; E = gain or loss in weight in grams during the critical period; F = weight of total food intake in grams during the depletion period; and $\bar{X}$ = estimated gain in grams during the test period.

The value of this equation for the estimation of future growth performance is illustrated by comparing estimated gains with the actual growth records of ten rats selected at random from the group of 577 rats (table 3). Since the equation was based on records of animals fed different levels of vitamin supplement, the actual comparison was not made until the predicted gain of each animal was adjusted to the mean gain of its own specific assay group by subtracting or

adding the difference between the mean gain of the specific group to which a rat belonged and that (34.8 gm.) of the entire lot of 577 rats. Since only one rat failed to behave according to the information furnished by the regression equation, the equation appears to be a valuable tool for predicting at the end of the depletion period whether average or erratic performance can be expected of any individual rat.[16]

We next determined the more variable of the animals among the 577 by substitution of data relating to each rat in the regression equation. When the range suggested by the mean

TABLE 3

Gains of individual female rats estimated at end of depletion period by means of the regression equation and the gains actually made in succeeding 8-week period of supplementary feeding

RAT NO.	ESTIMATED GAIN	ACTUAL GAIN
	gm.	gm.
6660	35	35
6676	42	53
6459	40	38
6486	34	30
6653	34	−11
6668	32	20
6423	37	34
6761	40	45
8861	36	48
9105	34	34
11126	15	6

gain ± the laboratory standard deviation was used for judging future behavior of an animal, erratic performance was estimated for 20% of the animals. We felt, however, that this range could be justifiably decreased because the inherent nature of the regression equation fixes the range in estimated gains below that of the actual gains. The range was therefore arbitrarily decreased and only those animals were considered suitable for testing whose gains fell within the range of the mean gain ± 5 gm. Fifty per cent of the 577 animals met this requirement, the standard deviation of their gains in the test period being 16.1 gm.

[16] The calculations involved in the estimation may be simplified in routine work by the construction of an alignment chart (Swett, '28).

Fisher ('37) has described a quantity known as invariance, that measures the information supplied by any particular experiment. In this case, $1/(18.1)^2$ has been taken as one unit of information because 18.1 represents the original standard deviation. After discarding the unsuitable rats, the amount of information yielded by the experiment was greater than before, for with the standard deviation dropping to 16.1, the information supplied equals $\frac{18.1^2}{16.1^2}$, or an increase of 26%.[17]

Of importance is the fact that the elimination of non-standard animals did not affect the normality of the distribution of the remaining data as shown by calculations that tested for skewness and kurtosis of the curve, nor did the removal of data pertaining to undesirable animals greatly alter the average gains of the ten lots of rats fed different dosages of vitamin A-carrying supplement. Results are shown in table 4.

However, the variability in every test lot but one (table 4) was decreased by the elimination of unpromising animals. In each of the lots, the standard deviation of the rats retained was smaller than the standard deviation of the original lot, whereas that of the rats eliminated was greater.

The statistic, 16.1, has been designated as the laboratory standard deviation. It becomes, therefore, the measure of the approximate variation that we shall expect to find in any assay conducted in our laboratory in the future under these conditions in an 8-week test period.

SHORTENING THE TEST PERIOD

In studies of gains made by various assay groups, a decrease in the weekly increments in weight often occurred after 35 days of supplementary feeding, especially when A was offered at one of the lower levels. This was associated with increased variability within the lot. Would the reliability of the test be improved in our case by a shortening of the experimental period? Other authors have proposed this step but with one or two exceptions (Coward, '33; Sherman and Burtis, '35), recommendations are not fortified by adequate studies.

[17] $I = \frac{V_1}{V_2}$, in which I represents information and V, the variance or the square of the standard deviation.

We now re-analyzed the data representing the growth induced in the original ten test lots on the basis of responses obtained in the first 5 weeks of the test period only. We

TABLE 4

Mean gain with standard deviation of ten lots of rats, and of the portions of each retained and discarded over test periods, 8 and 5 weeks long

LENGTH OF TEST PERIOD	SUPPLEMENTARY FOOD	ORIGINAL LOTS			GROUPS RETAINED			DISCARDED GROUPS		
		Number of rats	Mean gain	s	Number of rats	Mean gain	s	Number of rats	Mean gain	s
	mg.		*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*
8 weeks	Sweet potato									
	35	108	44.7	17.4	56	46.8	16.0	52	42.5	19.0
	30	40	45.6	19.4	21	39.8	18.9	19	52.1	20.4
	25	39	34.0	17.7	19	36.3	15.3	20	31.7	20.8
	20	17	33.1	20.0	10	32.5	16.0	7	41.4	29.6
	Tomato									
	175	27	50.7	13.1	22	52.4	12.5	5	43.6	17.7
	125	69	34.9	17.8	45	36.4	15.6	24	32.0	21.8
	100	56	17.9	20.2	30	22.1	18.4	26	15.6	22.6
	Carotene									
	0.001	22	50.4	19.5	7	48.6	23.3	15	50.7	19.2
	0.0005	36	28.4	16.9	16	34.6	15.7	20	23.6	18.2
	0.0004	23	16.6	17.7	11	18.8	14.0	12	14.7	21.0
5 weeks	Sweet potato									
	35	108	34.2	14.4	65	35.8	11.7	43	31.7	17.7
	50	40	35.4	15.4	19	32.2	14.8	22	38.1	16.2
	25	39	28.5	12.4	19	29.5	10.8	20	27.6	14.0
	20	17	29.8	17.2	9	24.4	14.8	8	35.6	19.2
	Tomato									
	175	27	38.0	7.4	18	37.8	6.5	9	38.2	8.9
	125	69	30.9	11.0	44	31.4	10.9	25	30.0	11.5
	100	56	21.9	17.9	30	24.8	14.6	25	13.6	20.4
	Carotene									
	0.001	22	40.5	13.4	14	41.4	16.0	8	39.0	7.8
	0.0005	36	25.6	11.2	18	29.8	10.4	18	21.4	12.3
	0.0004	23	18.1	15.5	12	23.3	9.2	11	12.4	20.9

found a marked improvement in the assay. The standard deviation was reduced from 18.6 to 13.9. The variations in growth response occurred in large part in the last few weeks of the long experimental period. No significant difference

could be demonstrated between the mean gains made by the animals in the 5- and 8-week periods, except in one case (table 5). Oser's statement ('35) that where steady minimal growth is a criterion the longer the test period the more consistent is the response apparently does not apply in our situation.

Several factors may account for the greater variability in mean increments of growth in the last weeks of the experiment. The variation in the mean weekly rates of growth increases in normal rats of the same approximate age of these assay rats

TABLE 5

Mean gains made by groups of depleted animals fed different sources of vitamin A for two specific intervals of time

SUPPLEMENTARY FOOD	NUMBER OF RATS PER GROUP	TIME SUPPLEMENT WAS FED				MEAN DIFFERENCES IN GAINS IN WEIGHT
		8 weeks		5 weeks		
		Mean gain in weight	s	Mean gain in weight	s	
		gm.	gm.	gm.	gm.	gm.
35 mg. sweet potato	108	44.7	17.4	34.2	14.4	10.5
30 mg. sweet potato	40	45.6	19.4	35.4	15.4	10.2
35 mg. sweet potato	39	34.0	17.7	28.5	12.4	5.5
20 mg. sweet potato	17	33.1	20.0	29.8	17.2	3.3
175 mg. tomato	27	50.7	13.1	38.0	7.4	12.7[1]
125 mg. tomato	69	34.9	17.8	30.9	11.0	4.0
100 mg. tomato	56	17.9	20.2	21.9	17.9	— 4.0
1.0 γ carotene	22	50.4	19.5	40.5	13.4	9.9
0.5 γ carotene	36	28.4	16.9	25.6	11.2	2.8
0.4 γ carotene	23	16.6	17.7	18.1	15.5	— 1.5

[1] This difference only is significant as shown by values of s.

(Timson, '32). Also, in the longer test period, the experiment extends beyond the linear portion of the normal growth curve. Finally, it is possible, especially with minimal doses of vitamin A, that inroads of infection do not manifest themselves until the later part of the experiment.

Coward ('33) analyzed growth response at various intervals and concluded that the increase in accuracy obtained by the prolongation of the vitamin A test beyond a period of 3 weeks was too slight to justify the extra expenditure and labor involved. Whether the test period can be shortened to less than 5 weeks in our laboratory as recommended by the U. S.

Pharmacopoeia Committee ('34) without sacrifice of accuracy will be tested later. The similarity in the standard deviation of the total gains at weekly intervals obtained in two different laboratories should be noted at this point. Coward ('33) found the standard deviation of the increase in weight to be 5.6, 8.1, 9.8, 11.1 and 11.9 gm. in succeeding weeks, whereas in one lot of our animals the standard deviations for 8 successive weeks were 3.8, 6.9, 6.8, 8.8, 10.9, 12.3, 14.5 and 17.8 gm.

To test the possibility of reducing the variability of the 5-week assay by the elimination of unsuitable animals, a new regression equation was calculated, i.e.,

$$\overline{X} = 76.88 - 0.28A - 1.31B - 0.23C - 1.23D - 0.36E + 0.09F$$

The data pertaining to each rat were substituted in the equation as before and those whose estimated gain varied from the mean (29.3 gm.) by more than $\pm$ 4 gm. were eliminated. The standard deviation dropped to 12.0 gm. The information now contributed by the experiment is $\frac{(18.1)^2}{(12.0)^2} = 2.27$ units in contrast to 1 unit in the original experiment, an increase of 127%. This means that in comparing test groups composed of fifteen to twenty animals, differences in mean gains of 7 to 8 gm. can be considered significant.[18] In the original assay, a difference of 11 gm. in the results of two assays being compared was necessary in order to make this interpretation. The figure, 12.0 becomes our laboratory standard deviation for a 5-week test period when the less desirable animals are eliminated.

PRACTICAL APPLICATION OF RECOMMENDED PROCEDURES

The studies herein presented show that a judicious handling of experimental data may markedly increase the uniformity and thence the reliability of an assay. We recommend first, the inclusion in the final assay of only a predetermined portion

[18] The following formula was used, $s_{MD} = \sqrt{\frac{s^2}{n_1} + \frac{s^2}{n_2}}$ where s_{MD} represents the average deviation of the mean difference in gains made by two test lots; s, the laboratory standard deviation; and n, the number of animals in each lot. The minimum mean difference for significance is $2s_{MD}$.

of the depleted rats. The elimination of rats of irregular behavior as estimated by means of the regression equation in no way affects the normality of the distribution of remaining data. Second, evidence has been presented that shows that the use of a 5-week test period is superior to one 8 weeks long. As a result, the standard deviation may be reduced from 18.1 gm. to 12.0 gm. with increase in the information supplied by the experiment of 127%. However, the usefulness and efficacy of technics of this kind must be fully tested. Several comparisons and tests were therefore made.

A comparison of the reliability of our assay with those of other investigators is interesting. As far as we know, Coward ('32 and '33) is the only other worker who has subjected as large a mass of data as ours to similar analysis. She found the average standard deviation of the gains of 672 females in an experimental period of 5 weeks to be 11.0 gm.; in a later test involving 1110 rats, 11.9 gm. Her animals are, therefore, less variable than are our unselected rats (standard deviation, 13.9). However, by selection, the variation in response of our rats can be reduced to 12.0 gm., showing that under these circumstances, assays from the two laboratories are more directly comparable than they were in the first instance.

The use of the regression equation as a measure for the identification of irregular animals also needed justification from the standpoint of results obtained in the analysis of data not represented in the regression equation. If the equation furnishes a reliable basis for the elimination of unsuitable animals, one-half of the animals in a new assay group should be found sub-standard at the end of the depletion period and the standard deviation from the mean gain made by the remaining animals in a 5-week assay period should approximate the laboratory standard deviation, i.e., 12.0 gm.

In order to test the hypothesis, a group of twenty-eight animals were depleted of vitamin A in the standard routine manner 1 year after the equation was calculated. Upon substitution of individual data (table 6) in the regression equation, erratic response was predicted of fifteen rats, the esti-

TABLE 6

Example of reduction of size of an experimental lot at the end of the depletion period on the basis of a suitable regression

TABLE 6

Example of reduction of size of an experimental lot at the end of the depletion period on the basis of a suitable regression equation

Regression equation: $\bar{X} = 76.88 - 0.28A - 1.31B - 0.23C - 1.23D - 0.36E + 0.09F$ (5 weeks)

GROUPS FORMED	RAT NO.	A WEIGHT AT WEANING	B LENGTH OF DEPLETION PERIOD	C GAIN IN WEIGHT IN DEPLETION PERIOD	D TIME OF CONSTANT WEIGHT OF DEPLETION PERIOD	E GAIN OR LOSS IN WEIGHT IN CRITICAL PERIOD	F FOOD CONSUMED IN DEPLETION PERIOD	$\bar{X}$ ESTIMATED GAIN IN TEST PERIOD	X ACTUAL GAIN IN TEST PERIOD
		gm.	*days*	*gm.*	*days*	*gm.*	*gm.*	*gm.*	*gm.*
Animals eliminated	13777	44	28	54	5	− 2	152	23.7	
	13807	42	30	50	5	2	175	23.2	
	13886	40	28	63	5	3	197	25.0	
	14041	41	30	71	5	2	215	22.2	
	13466	38	32	62	5	1	187	20.4	
	13474	39	26	61	5	0	149	25.1	
	14520	35	14	17	5	2	58	43.2	
	13908	46	17	14	5	3	96	39.9	
	14124	50	28	62	5	2	194	22.5	
	11338	48	36	92	4	2	267	13.5	
	14039	36	29	76	5	4	214	23.0	
	13783	44	29	60	4	1	169	22.7	
	13862	40	32	66	5	3	204	19.7	
	13836	36	29	62	6	1	179	22.9	
	13808	42	30	53	5	0	178	23.5	
Animals retained	13801	30	23	50	6	4	131	29.8	21
	13645	44	21	49	5	3	131	30.3	31
	13722	46	22	53	5	2	129	27.7	46
	13747	36	22	42	5	2	114	31.7	42
	13955	40	25	56	5	4	147	25.7	38
	13909	44	24	41	4	− 4	139	32.7	29
	14099	42	22	62	5	4	148	27.7	31
	14066	37	27	58	5	3	171	26.0	26
	14064	36	25	66	5	2	171	27.4	15
	14047	31	27	53	5	2	175	29.5	18
	14046	44	29	59	3	1	193	26.3	17
	13482	42	25	54	5	4	162	26.9	12
	13473	41	24	51	4	− 1	119	28.4	32

mated response of all of these falling outside the range, 29.3 ± 4 gm.

The thirteen remaining rats were fed 0.0006 mg. of carotene in the test period. The mean gain of this group was 27.5 gm., with a standard deviation of 10.5 gm. The agreement of the resulting standard deviation with that discussed above was gratifying. This test also suggested that our stock colony did not represent a shifting population. Otherwise, the laboratory standard deviation could not be used for estimating the reliability of a test.

We wondered also whether undesirable animals could be identified in tests run before the assay was as highly standardized as it is at present. An assay conducted in 1930 in which rats received 30 mg. of butterfat as the sole source of vitamin A was therefore re-analyzed. The mean gain of the original group over an experimental period of 5 weeks was 18.7 gm. with a standard deviation of 17.1 gm. In scanning the data pertaining to this test (table 7), casual inspection shows in the light of our present criteria, that at least twelve animals were over-depleted. Upon testing with the regression equation, these rats were in the group estimated as erratic. The mean gain made by the irregular animals was 13.6 gm., that of the remaining group, 30.4 gm. The standard deviations were 17.7 gm. and 8.9 gm., respectively. Probably 30.4 gm. more nearly represents the true response of depleted rats to the daily administration of this quantity of butterfat than does the result of the original analysis, and is one less colored by pathologic influences.

The recommended procedures also are of direct practical importance. Estimations showed that both the time involved for the completion of an assay and the number of animals needed for the actual test may be lowered in the neighborhood of 40%. Laboratory expense is also cut a corresponding degree.

If an investigator does not feel justified in actually eliminating animals at the end of the depletion period and in shortening the test period as suggested herein, he may try the effect for himself without disturbing his usual procedure.

TABLE 7

Estimated and actual gains made by desirable and undesirable animals in a lot composed of twenty-three rats, in the main, over-depleted

Regression equation: $\overline{X} = 76.88 - 0.28A - 1.31B - 0.23C - 1.23D - 0.36E + 0.09F$ (5 weeks)

GROUPS FORMED	RAT NO.	A WEIGHT AT WEANING	B LENGTH OF DEPLETION PERIOD	C GAIN IN WEIGHT IN DEPLETION PERIOD	D TIME OF CONSTANT WEIGHT OF DEPLETION PERIOD	E GAIN OR LOSS IN WEIGHT IN DEPLETION PERIOD	F FOOD CONSUMED IN DEPLETION PERIOD	$\overline{X}$ ESTIMATED GAIN IN TEST PERIOD	X ACTUAL GAIN IN TEST PERIOD
		gm.	*days*	*gm.*	*days*	*gm.*	*gm.*	*gm.*	*gm.*
Undesirable animals	6153	42	22	48	4	− 2	145	34.1	33
	6164	50	29	62	7	2	221	21.2	15
	6225	60	30	47	12	3	209	12.9	24
	6322	52	21	49	5	− 5	171	34.6	19
	6205	40	31	54	9	4	190	17.2	11
	6239	40	33	74	8	4	233	15.1	14
	6169	55	32	77	9	5	247	11.2	27
	6213	51	25	49	7	3	166	23.8	17
	6262	54	35	63	8	4	299	10.7	− 15
	6220	47	32	60	11	4	209	11.8	− 4
	6163	48	33	77	5	5	218	14.2	17
	6155	46	36	78	9	8	263	10.1	− 24
	6238	45	28	59	6	0	181	22.9	36
	6224	56	28	54	7	3	191	19.6	− 2
	6156	40	21	44	3	− 3	129	37.1	40
	6211	44	26	78	7	0	180	20.2	9
Desirable animals	6319	58	21	45	5	1	158	30.5	28
	6329	45	21	52	5	2	144	30.9	32
	6327	52	21	47	5	0	150	31.3	33
	6321	55	21	43	5	− 2	158	32.8	39
	6161	47	25	67	3	3	198	28.6	27
	6154	42	22	59	4	− 1	153	31.9	36
	6221	45	25	48	6	3	167	27.1	18

Let him compute the expected gains of his animals, designating in advance the half of the animals expected to make the least erratic gains. He can then compute results on both the half and the entire lot, thus learning if his results compare with ours, but without risk of sacrificing his test.

SUMMARY

In a study of methods for the improvement of the bio-assay for vitamin A, the average standard deviation from the mean gain has been chosen as the criterion in making comparisons.

The possibility of improving the bio-assay for vitamin A by strict laboratory standardization of the generally accepted method has been demonstrated.

Further reduction of the variability of the assay has been effected through two channels. First, animals were eliminated from the assay group that gave indications in the depletion period of erratic response to the feeding of the test substance. These eliminations were made on the basis of estimations of future gain by means of a regression equation calculated on certain data collected in the depletion period and later gain in the test period. Second, the uniformity and thence the reliability of the assay has been increased by shortening the test period from 8 to 5 weeks.

The adoption of the two procedures in conducting the assay increased the information yielded by the test 127%.

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THE INFLUENCE OF DIET ON THE NITROGEN BALANCES OF PRE-SCHOOL CHILDREN [1]

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TWO FIGURES

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Although there has been much discussion in the literature concerning the value of high and low protein diets, the protein neeeds of pre-school children are still questioned. Experimental evidence suggests that children might develop faster and be physically stronger if they had a high rather than a low protein diet. These advantages may arise not only from an increase in protein content, but also from a proper balance between the protein and the other constituents in the diet or between the various essential amino acids. At the present time, it is impossible to prepare an adequate synthetic diet for children from different amino acids. Thus the value of protein must be determined by varying its amount and not by varying the amounts of different amino acids in the diet. In previous studies, the experimental periods were exceedingly short or there were several variables beside the amount of protein in the diet. Therefore, the purposes of the present study were, first, to determine the variations in the nitrogen balances of the pre-school children who received a constant diet and, second, to find the changes in the nitrogen balances when only the protein content of the diet was changed.

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Previous investigators do not agree as to the exact amount of protein needed. Sherman ('37) says that the growing child should have 10% of the total caloric intake in the form of protein or more than twice as much per unit of weight as the adult. According to Rose ('33) this amount would be approximately 3 gm. per kilogram. Since the calorie intake of a child varies in relation to his activity, a standard based on percentage of calories eaten may be quite variable. Therefore, the question arises as to the exact amount necessary for optimum nutrition. Several investigators have determined the minimum quantity necessary. Parsons ('30) says that normal children from 4 to 8 years of age can maintain a positive nitrogen balance on 0.5 to 1.1 gm. of protein per kilogram, if the caloric intake is adequate. Bartlett ('26) found that diabetic children needed practically the same amount, 0.6 to 1.0 gm. per kilogram, while Boyd ('25) could not obtain positive nitrogen balances on less than 1.25 gm. of protein per kilogram. Although these subjects on low nitrogen intakes did gain weight, in the light of the studies which Wang, Hawks and Hayes ('28), Wang, Hawks and Kaucher ('28), Wang, Kern and Kaucher ('29) made on undernourished children, their nutritional development may not have been optimal. These authors ('29) noted positive nitrogen balances on low levels of nitrogen intake, but higher retentions and greater gains in weight following an increase in protein content of the diet. Daniels and her co-workers ('35) in their study on the protein needs of pre-school children, found that, based on creatinine elimination in terms of theoretical weight, the children retained the greatest amount of nitrogen when they received approximately 3.2 gm. of good quality protein per kilogram. Although these studies have added valuable information to our knowledge of protein metabolism, all of the factors which influence the utilization are not known.

The results of the present study may give further information because the period of study was fairly long and there were few variations in the procedure (Hawks, Bray and Dye, '37 a). Six normal pre-school children received two adequate

diets, one containing 3 and the other 4 gm. of protein per kilogram of body weight. The first diet continued for 21 days and the second for 15 or 24 days. Although the authors took every precaution to have a constant diet, there were slight fluctuations not only from period to period but also within a period. In the first experiment on two children, the calories were constant but the protein was not the only variable because skimmed milk, egg and beef raised the protein content. In the second experiment on four children, there were fewer variables, for egg white and gelatin increased the protein and the omission of butter kept the calories at the same level. These changes in diet increased the amount of protein from animal sources 8.5 and 6.8% in the two experiments, respectively. Although Belousov and Gilman ('34) found that children utilized animal protein better than vegetable protein, the differences in the present study were probably too slight to influence the results. Some investigators (Edelstein, Langer and Langstein, '31; Edelstein, '32, and Funnell and her associates, '36) found that an increase in bulk content of the diet decreased protein utilization, while Schultz, Morse and Oldham ('33) said that small increases in bulk had little influence. However, in this experiment, the bulk content could not have been an important factor because there was a difference of only 12.6 gm. of vegetable between the two diets in the first experiment and none in the second. According to a number of investigators (Weber, '32; Krause, '33; Davis, '35, and Röse, '35) who studied the effect of acid and basic diets on the nitrogen retention of infants and children, protein was most effectively utilized when the diet residue was basic. All of the diets in the present study were basic, the medium and high protein diets varying only 8.4 and 4.1 cc. of normal solution in the first and second experiments, respectively. Therefore, the acid base relationship was probably not an influential factor. The change in the calcium and phosphorus content of the diet was negligible in the second experiment, but, in the first, the calcium increased 0.168 gm. and the phosphorus 0.313 gm. Protein, therefore,

TABLE 1

Nitrogen balance data on a constant medium protein diet, expressed per kilogram of body weight

CHILD	EXPERIMENT	PERIOD	INTAKE		OUTPUT									ABSORPTION		RETENTION	
					Urine		Feces					Total					
			Nitrogen	Dried weight	Total nitrogen	Proportion intake	Total nitrogen	Proportion intake	Nitrogen per gram dried food	Dried weight	Nitrogen per gram dry feces	Total nitrogen	Proportion intake	Total nitrogen	Proportion intake	Total nitrogen	Proportion intake
			mg.	*gm.*	*mg.*	%	*mg.*	%	*mg.*	*gm.*	*mg.*	*mg.*	%	*mg.*	%	*mg.*	%
B	I	1	506	16.9	389	76.8	78	15.5	4.63	1.31	60	467	92.3	428	84.5	39	7.7
		2	504	17.1	379	74.6	103	20.4	6.02	1.42	73	479	95.0	401	79.6	25	5.0
		3	526	17.2	402	76.4	70	13.3	4.08	1.17	60	472	89.7	456	86.7	54	10.3
		4	533	17.4	418	78.4	63	11.8	3.63	1.10	57	481	90.2	470	88.2	52	9.8
		5	500	17.3	405	80.9	63	12.6	3.67	1.18	53	468	93.5	437	87.4	32	6.5
		6	518	16.8	416	80.4	63	12.2	3.75	1.13	56	479	92.6	455	87.8	39	7.4
		7	507	16.9	412	81.4	63	12.4	3.71	1.10	57	476	93.8	444	87.6	31	6.2
		Mean	513	17.1	403	78.4	72	14.0	4.21	1.20	60	475	92.4	441	86.0	38	7.6
D	I	1	501	16.7	412	82.4	64	12.8	3.83	1.30	49	476	95.2	437	87.2	25	4.8
		2	500	16.9	419	83.8	60	12.0	3.54	1.21	50	479	95.8	440	88.0	21	4.2
		3	521	17.0	436	83.7	46	8.8	2.71	0.97	47	482	92.5	475	91.2	39	7.5
		4	526	17.1	434	82.5	49	9.3	2.84	1.03	48	483	91.8	477	90.7	43	8.2
		5	492	17.0	425	86.3	48	9.8	2.83	1.21	40	473	96.1	444	90.2	19	3.9
		6	513	16.7	444	86.6	51	9.9	3.04	1.05	49	495	96.5	462	90.1	18	3.5
		7	502	16.8	421	83.9	53	10.4	3.12	1.11	48	474	94.3	449	89.6	29	5.7
		Mean	508	16.9	427	84.2	53	10.4	3.13	1.13	47	480	94.6	455	89.0	28	5.4

		Mean	508	16.9	427	84.2	53	10.4	3.13	1.13	47	480	94.6	455	89.6	28	5.4
V	II	1	439	18.0	393	89.5	33	7.5	1.83	0.78	42	426	97.0	406	92.5	13	3.0
		2	454	17.4	396	87.2	32	7.1	1.84	0.79	41	428	94.3	422	92.9	26	5.7
		3	...	...	...	...	..	...	...	...	..	...	....	...	....	..	....
		4	...	...	...	...	..	...	...	...	..	...	....	...	....	..	....
		5	454	17.6	395	87.0	32	7.1	1.84	0.83	39	427	94.1	422	92.9	27	5.9
		6	466	17.8	402	86.4	33	7.1	1.87	0.80	41	435	93.5	433	92.9	31	6.5
		7	436	17.7	374	85.9	35	7.9	1.95	0.77	45	409	93.8	401	92.1	27	6.2
		Mean	450	17.7	392	87.2	33	7.3	1.87	0.79	42	425	94.5	417	92.7	25	5.5
C	II	1	456	18.7	398	87.5	44	9.6	2.35	0.91	48	442	97.1	412	90.4	14	2.9
		2	473	18.1	398	84.2	46	9.7	2.54	0.92	50	444	93.9	427	90.3	29	6.1
		3	488	18.5	396	81.2	45	9.2	2.44	0.90	50	441	90.4	443	90.8	47	9.6
		4	482	18.5	403	83.6	45	9.4	2.45	0.92	49	448	93.0	437	90.6	34	7.0
		5	471	18.3	397	84.3	45	9.6	2.48	0.94	48	442	93.9	426	90.4	29	6.1
		6	484	18.4	400	82.6	46	9.6	2.51	0.91	51	446	92.2	438	90.4	38	7.8
		7	452	18.3	379	83.9	45	10.0	2.48	0.89	51	424	93.9	407	90.0	28	6.1
		Mean	472	18.4	396	83.9	45	9.6	2.46	0.91	49	441	93.5	427	90.4	31	6.5
J	II	1	427	17.5	356	83.4	52	12.1	2.95	0.92	57	408	95.5	375	87.9	19	4.5
		2	441	16.9	363	82.2	54	12.2	3.18	0.93	55	417	94.4	387	87.8	24	5.6
		3	456	17.3	365	80.1	57	12.4	3.28	1.01	56	422	92.5	399	87.6	34	7.5
		4	453	17.4	363	80.1	59	13.0	3.40	1.07	55	422	93.1	394	87.0	31	6.9
		5	443	17.2	374	84.7	59	13.0	3.44	1.07	55	433	97.7	384	87.0	10	2.3
		6	454	17.3	367	80.8	54	11.9	3.13	0.99	55	421	92.7	400	88.1	33	7.3
		7	423	17.1	342	80.8	54	12.8	3.15	0.99	55	396	93.6	369	87.2	27	6.4
		Mean	442	17.2	361	81.7	56	12.5	3.22	1.00	56	417	94.2	386	87.5	25	5.8

was the chief variable in the two similar experiments, since the percentage of protein from animal sources, the bulk, the acid-base relationship, calcium, phosphorus and calories were practically constant.

EXPERIMENTAL RESULTS

Medium protein diet. The reactions of each child to the standardized medium protein diet was remarkably constant from period to period, but there were some variations in the reactions of different children. Table 1 and figure 1 show

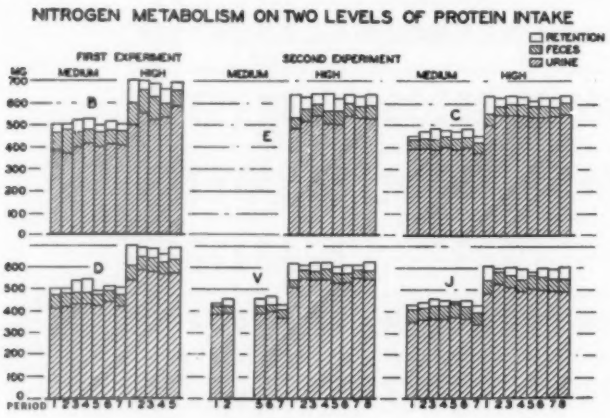

Figure 1

the nitrogen balances for the children who received this diet and table 2 gives the statistical evaluation of the data.

For each child, the total nitrogen output as well as the figures for urine and feces varied little from period to period. The average percentage of the intake excreted both in the urine and feces varied between the children, but the values seemed to compensate each other so that the total excretion represented between 92.4 and 94.6% of the intake. Considering the data for experiments one and two rather than for individual children, Hawks, Bray and Dye ('37 b) previously reported that the variations in diet nitrogen influenced urinary

excretion because the coefficients of variation were similar. Table 2 shows that the coefficients of variation for total output were also practically the same as those for diet and that, in the second experiment, the correlation with intake was high. Feces values apparently had no relation to intake variation and the average figures were quite different for individual children. Therefore, the practically constant diet nitrogen must have caused the children to excrete the same proportion

TABLE 2

Statistical evaluation of nitrogen data on 3-gm. protein diet

	EXPERIMENT	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION	CORRELATION WITH INTAKE	CORRELATION WITH DRY WEIGHT OF FOOD	CORRELATION WITH DRY WEIGHT OF FECES
		mg.	*mg.*				
Intake	I	511 ± 1.2	12.0	2.3	....	....	....
	II	457 ± 1.5	17.2	3.8	....	....	....
Output							
Urine	I	415 ± 2.0	19.5	4.7	0.21	....	....
	II	384 ± 1.6	18.6	4.8	0.67	....	....
Feces	I	62 ± 2.7	14.8	23.7	— 0.10	0.15	0.75
	II	46 ± 1.4	9.3	20.2	— 0.15	— 0.35	0.91
Total	I	477 ± 1.3	7.0	1.5	0.36	....	....
	II	428 ± 2.2	14.3	3.3	0.84	....	....
Absorption	I	448 ± 3.6	20.2	4.5	0.64	....	....
	II	410 ± 3.4	21.9	5.4	0.86	....	....
Retention	I	33 ± 2.1	11.5	34.6	0.77	....	....
	II	27 ± 1.4	8.9	32.4	0.63	....	....

of the intake, but some other factors must have produced the differences in the distribution between the urine and feces. Although the bulk and other diet factors remained constant, the character of the stools varied. Subjects D., B. and J. had rather loose stools while V. had quite constipated ones. Consequently, the dry weight of the stools (table 1) varied between different children but showed high correlations with the feces nitrogen, 0.75 and 0.91 (table 2). Although these subjects did not receive a low nitrogen diet, the results indicated, as did those of Heupke ('34) and Heupke and Belz ('35), that

fecal nitrogen was dependent upon the weight of the feces and that there may have been a constant nitrogen fraction excreted. Schneider ('35), however, noted that with human subjects there seemed to be no constant fraction for metabolic nitrogen, all of it varying in proportion to the intake of dry food. The ratio of fecal nitrogen to dry matter consumed (table 1) showed that this may have been true for some individual children, but, the average ratios for the different children varied according to the nature of the stools, and there was no correlation between the two factors. The ratios were within the range of those which Mitchell ('26) reported for human subjects receiving low nitrogen diets, but they were lower than those which Schneider reported, possibly because the bulk content of the present diet must have been lower than that which he used. Nevertheless, the data indicate that there were individual variations in fecal nitrogen excretion which were not shown in the total excretion.

Since the feces values varied for individual children, the absorption or coefficient of digestibility of the same food varied from 86.0 to 92.7% of the intake. These values were quite constant for each individual child and they showed a high degree of correlation with intake, especially in the second experiment (table 2). The small variations in intake were also reflected in the retentions of these children who were in nitrogen equilibrium. All of the retentions were positive and, considering the number of biological and technical errors, they were remarkably constant, the average values representing from 5.4 to 7.6% of the intake. In spite of the high coefficients of variation in the retention values, there was a definite correlation with intake, 0.77 and 0.63 for the two experiments. Therefore, the data on the medium protein diet show that the children reacted in a similar manner and retained nitrogen in proportion to the period by period variations in diet, even though they did not excrete the same proportion of nitrogen in urine and feces.

High protein diet. The reactions of the children to the high protein diet were similar to those on the medium protein diet.

Yet there were some differences in the amounts and in the proportions excreted both by way of the urine and feces. Figure 1 as well as table 3 show that the total output was higher for each child on every period. During the first period following the change in diet, there was a definite lag in the urinary nitrogen excretion. The values were above those on the medium protein diet but lower than any of the other high protein figures. Since the fecal nitrogen values were not proportionally higher during the first period, the total excretions were low and the retentions high. Although the values during the second and third periods were similar to all others, a preliminary period of 9 days, as used for the data on the urinary nitrogenous constituents (Hawks, Bray and Dye, '37 b), certainly covered the fluctuations due to change in diet. These preliminary values were less constant than those for the medium protein diet or for all of the high protein diet because the statistical evaluation of the data (table 4) showed higher standard deviations and coefficients of variability, and less correlation between intake and the several functions.

In the first experiment, there were only two periods following the preliminary period. Therefore, the differences in the metabolism of the children on the two diets can best be determined by comparing the data for the last five high protein periods in the second experiment with the data for the same children on the medium protein diet. A comparison of these data after the children had reached equilibrium on the two levels of protein intake indicated that there were slight differences in nitrogen utilization (table 5). The percentage of the intake excreted in the urine was higher on the high protein diet, while that excreted in the feces was lower, but again these two values seemed to compensate each other so that the total excretion represented approximately the same constant percentage of the intake that it had on the medium protein diet, from 90.1 to 93.0%. As before, the fecal nitrogen seemed to vary according to the character of the stools, but the nitrogen increase was less than the increase in diet nitrogen and

TABLE 3

Nitrogen balances of children who received a constant diet containing 4 gm. of protein per kilogram

CHILD	EXPERIMENT	PERIOD	INTAKE		OUTPUT									ABSORPTION		RETENTION	
					Urine		Feces					Total					
			Nitrogen	Dried weight	Total nitrogen	Per cent intake	Total nitrogen	Per cent intake	Nitrogen per gram dried food	Dried weight	Nitrogen per gram dry feces	Total nitrogen	Per cent intake	Total nitrogen	Per cent intake	Total nitrogen	Per cent intake
			mg	*gm.*	*mg.*	%	*mg.*	%	*gm.*	*gm.*	*mg.*	*mg.*	%	*mg.*	%	*mg.*	%
B	I	1	707	17.7	503	71.2	101	14.3	5.72	1.54	66	604	85.5	606	85.7	103	14.5
		2	704	17.3	558	79.3	105	14.9	6.06	1.48	71	663	94.2	599	85.1	41	5.8
		3	691	18.5	529	76.5	104	15.1	5.63	1.44	72	633	91.6	587	84.9	58	8.4
		4	668	18.1	540	80.9	59	8.8	3.24	1.11	53	599	89.7	609	91.2	69	10.3
		5	696	16.0	587	84.3	73	10.5	4.58	1.30	56	660	94.8	623	89.5	36	5.2
	Mean	1–3	701	17.9	530	75.6	103	14.8	5.80	1.49	69	633	90.4	598	85.2	68	9.6
	Mean	All	693	17.5	543	78.4	88	12.8	5.05	1.37	64	631	91.2	605	87.2	62	8.8
D	I	1	699	17.5	542	77.6	67	9.5	3.80	1.23	55	609	87.1	632	90.5	90	12.9
		2	696	17.2	589	84.7	62	9.8	3.60	1.17	53	651	93.6	634	91.1	45	6.4
		3	687	18.4	581	84.6	65	9.5	3.55	1.21	54	646	94.1	622	90.5	41	5.9
		4	662	17.9	570	86.0	57	8.6	3.18	1.09	52	627	94.6	605	91.4	35	5.4
		5	690	15.8	576	83.4	62	9.0	3.91	0.88	71	638	92.4	628	91.0	52	7.6
	Mean	1–3	694	17.7	571	82.3	65	9.3	3.65	1.20	54	636	91.6	629	90.7	58	8.4
	Mean	All	687	17.4	572	83.2	63	9.1	3.61	1.11	57	635	92.3	624	90.9	52	7.7
E	II	1	645	18.8	488	75.7	52	8.1	2.78	1.00	52	540	83.8	593	91.9	105	16.2
		2	637	18.8	524	82.4	55	8.5	2.88	1.00	55	579	90.9	582	91.5	58	9.1
		3	648	18.7	551	85.1	53	8.1	2.82	1.04	51	604	93.2	595	91.9	44	6.8
		4	646	19.1	516	79.9	57	8.7	2.95	1.06	54	573	88.6	589	91.3	73	11.4
		5	625	19.0	514	82.2	60	9.6	3.14	1.12	54	574	91.8	565	90.4	51	8.2
		6	642	19.3	548	85.3	59	9.2	3.08	1.09	54	607	94.5	583	90.8	35	5.5
		7	638	17.6	541	84.8	54	8.5	3.10	1.07	51	595	93.3	584	91.5	43	6.7
		8	649	18.9	538	82.8	59	9.1	3.15	1.05	56	597	91.9	590	90.0	52	8.1
	Mean	1–3	643	18.8	521	81.1	53	8.2	2.83	1.01	53	574	89.3	590	91.8	69	10.7
	Mean	4–8	640	18.8	531	83.0	58	9.0	3.08	1.08	54	589	92.0	582	91.0	51	8.0
	Mean	All	641	18.8	528	82.3	56	8.7	2.99	1.05	53	584	91.0	585	91.3	57	9.0
V	II	1	622	18.1	508	81.6	36	5.9	2.01	0.80	45	544	87.4	586	94.1	78	12.6
		2	614	18.3	551	89.7	40	6.6	2.22	0.89	45	591	96.3	574	93.4	23	3.7
		3	625	18.0	546	87.4	39	6.2	2.16	0.88	44	585	93.6	586	93.8	40	6.4

	Mean	4–8	640	18.8	531	83.0	58	9.0	3.08	1.08	54	589	92.0	582	91.0	51	8.0
	Mean	All	641	18.8	528	82.3	56	8.7	2.99	1.05	53	584	91.0	585	91.2	57	9.0
V	II	1	632	18.1	508	81.5	56	8.9	3.01	0.80	48	544	87.4	586	94.1	78	12.6
		2	614	18.2	551	89.7	40	6.6	2.32	0.89	45	591	96.3	574	93.4	23	3.7
		3	625	18.0	546	87.4	39	6.2	2.16	0.88	44	585	93.6	586	93.8	40	6.4
		4	623	18.5	548	87.9	48	7.7	2.61	0.99	49	596	95.6	575	92.3	27	4.4
		5	603	18.3	528	87.5	47	7.8	2.59	0.91	52	575	95.3	556	92.2	28	4.7
		6	616	18.5	533	86.5	41	6.7	2.23	0.86	48	574	93.2	575	93.3	42	6.8
		7	616	17.0	552	89.6	39	6.3	2.30	0.84	46	591	95.9	577	93.7	25	4.1
		8	627	18.2	549	87.6	40	6.3	2.18	0.82	49	589	93.9	587	93.7	38	6.1
	Mean	1–3	620	18.1	535	86.2	38	6.2	2.13	0.86	44	573	92.4	582	93.8	47	7.6
	Mean	4–8	617	18.1	542	87.8	43	7.0	2.38	0.88	49	585	94.8	574	93.0	32	5.2
	Mean	All	618	18.1	539	87.2	41	6.7	2.29	0.87	47	580	93.9	577	93.3	38	6.1
C	II	1	643	18.8	503	78.3	56	8.7	2.98	0.99	57	559	87.0	587	91.3	84	13.0
		2	634	18.7	551	86.9	41	6.5	2.20	0.83	49	592	93.4	593	93.5	42	6.6
		3	641	18.5	553	86.2	47	7.4	2.56	0.99	48	600	93.6	594	92.6	41	6.4
		4	638	18.9	548	85.9	52	8.2	2.77	1.05	49	600	94.1	586	91.8	38	5.9
		5	619	18.8	641	87.3	51	8.2	2.71	0.98	52	592	95.5	568	91.8	27	4.5
		6	634	19.0	541	85.3	51	8.0	2.67	0.98	52	592	93.3	583	92.0	42	6.7
		7	629	17.4	544	86.5	45	7.2	2.61	0.90	50	589	93.7	584	92.8	40	6.3
		8	641	18.6	558	87.0	49	7.7	2.65	0.89	55	607	94.7	592	92.3	34	5.3
	Mean	1–3	639	18.7	536	83.8	48	7.5	2.58	0.94	51	584	91.3	591	92.5	55	8.7
	Mean	4–8	632	18.5	546	86.3	50	7.9	2.68	0.96	52	596	94.2	582	92.1	36	5.8
	Mean	All	635	18.6	542	85.4	49	7.7	2.64	0.95	52	591	93.1	586	92.3	44	6.9
J	II	1	605	17.6	480	79.4	61	10.1	3.48	1.02	60	541	89.5	544	89.9	64	10.5
		2	596	17.6	523	87.8	57	9.6	3.26	1.00	57	580	97.4	539	90.4	16	2.6
		3	599	17.3	506	84.3	59	9.9	3.43	1.01	58	565	94.2	540	90.1	34	5.8
		4	595	17.6	488	82.1	56	9.3	3.15	0.96	58	544	91.4	539	90.7	51	8.6
		5	579	17.6	500	86.3	61	10.5	3.45	1.02	60	561	96.8	518	89.5	18	3.2
		6	593	17.8	497	83.7	59	10.0	3.33	1.04	57	556	93.7	534	90.0	37	6.3
		7	590	16.2	491	83.2	59	10.0	3.65	1.04	57	550	93.2	531	90.0	40	6.8
		8	600	17.4	489	81.5	59	9.9	3.41	1.04	57	548	91.4	541	90.1	52	8.6
	Mean	1–3	600	17.5	503	83.8	59	9.9	3.39	1.01	58	562	93.7	541	90.1	38	6.3
	Mean	4–8	591	17.3	493	83.4	59	9.9	3.40	1.02	58	552	93.3	532	90.1	39	6.7
	Mean	All	595	17.4	497	83.5	59	9.9	3.40	1.02	58	556	93.4	536	90.1	39	6.6

TABLE 4

Statistical evaluation of nitrogen data on 4-gm. protein diet

	PERIOD OF EXPERIMENT	PERIOD	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION	CORRELATION WITH INTAKE	CORRELATION WITH DRY WEIGHT OF FOOD	CORRELATION WITH DRY WEIGHT OF FECES
			mg.	*mg.*				
Intake	I	All	691 ±1.7	13.7	2.0	....	....	....
		1–3	697 ± 1.1	7.1	1.0	....	....	....
	II	All	622 ± 1.4	19.9	3.2	....	....	....
		1–3	626 ± 2.3	18.5	3.0	....	....	....
		4–8	620 ± 1.8	20.5	3.3	....	....	....
Output								
Urine	I	All	558 ± 4.1	32.7	5.9	— 0.21	....	....
		1–3	551 ± 5.7	36.0	6.5	0.43	....	....
	II	All	527 ± 1.9	27.2	5.2	0.53	....	....
		1–3	524 ± 3.9	31.3	6.0	0.32	....	....
		4–8	528 ± 2.2	25.1	4.8	0.68	....	....
Feces	I	All	76 ± 4.2	19.7	26.1	0.56	0.18	0.79
		1–3	84 ± 5.7	21.3	26.3	0.39	....	....
	II	All	51 ± 0.9	7.7	15.1	— 0.20	— 0.04	0.86
		1–3	50 ± 1.7	8.7	17.5	— 0.18	....	....
		4–8	52 ± 1.1	7.1	13.7	— 0.17	0.05	0.85
Total	I	All	633 ± 4.9	23.0	3.6	0.26	....	....
		1–3	634 ± 6.5	23.7	3.7	— 0.27	....	....
	II	All	578 ± 2.5	21.0	3.6	0.53	....	....
		1–3	573 ± 4.5	23.0	4.0	0.20	....	....
		4–8	581 ± 3.0	19.9	3.4	0.77	....	....
Absorption	I	All	615 ± 3.3	15.7	2.6	0.13	....	....
		1–3	613 ± 5.2	19.0	3.1	0.10	....	....
	II	All	571 ± 2.7	22.6	4.0	0.91	....	....
		1–3	576 ± 4.3	22.0	3.8	0.85	....	....
		4–8	578 ± 3.5	23.0	4.1	0.91	....	....
Retention	I	All	57 ± 5.0	23.5	41.2	0.31	....	....
		1–3	63 ± 7.4	27.0	42.9	0.46	....	....
	II	All	44 ± 2.3	19.4	43.8	0.41	....	....
		1–3	52 ± 5.1	26.2	50.1	0.47	....	....
		4–8	40 ± 1.9	12.4	31.1	0.34	....	....
Retention 3 children	II	All	40 ± 2.3	16.7	41.6	0.29	....	....
		1–3	47 ± 5.3	23.5	50.2	0.43	....	....
		4–8	36 ± 1.7	9.5	26.5	0.58	....	....

practically the same as the increase in dry weight of the feces. Therefore, the nitrogen per gram of dried feces decreased, and there was still a correlation of 0.85 between the two sets of data. Although the only differences in food were the addition of egg white and gelatin and the omission of butter, the dry weight of the food increased slightly, but not in proportion to the fecal nitrogen increase because the fecal nitrogen per gram of dried food was higher and there was still no correlation between these two factors. Thus these data seem to indicate that there must have been a constant nitrogen fraction excreted in the feces.

The high protein diet increased the coefficient of digestibility or the percentage of the intake absorbed as much as 2.6%, but caused less than 1.0% difference in the amount retained (table 5). The increase in absorption as well as the differences in fecal nitrogen may have been due to the fact that gelatin and egg white had higher biological values than the other proteins in the diet. It was not due to an increase in the variability in the data for the coefficient of variation was no higher. Fluctuation in diet nitrogen apparently caused the variations in absorption because the correlation between the two factors was 0.91. Since the retention values represented a proportion of the intake similar to that on the medium protein diet, the children actually stored more grams of nitrogen. These values were no more variable than they had been on the first diet, but the correlation with intake was low. This may have been caused by the high values for subject E. Since his data were not included in those for the medium protein diet and since he may have been storing more nitrogen on account of his previous illness, table 4 also gives the statistical data on the figures for the other three children. On this basis, the correlation between intake and retention was practically the same as that on the medium protein diet. Thus, after equilibrium, the three children on the second experiment stored nitrogen in relation to intake variations.

During the high protein diet all of the children gained at a faster rate than they had during the medium protein diet.

TABLE 5

Differences per kilogram of body weight between metabolism data after equilibrium on medium and high protein diets

SUBJECT	DIET			OUTPUT								ABSORPTION		RETENTION	
				Urine		Feces				Total					
	Nitrogen	Increase	Dry weight	Nitrogen	Proportion of intake	Nitrogen	Proportion of intake	Nitrogen per gram dried food	Dried weight	Nitrogen per gram dried feces	Nitrogen	Proportion of intake	Nitrogen	Proportion of intake	Nitrogen	Proportion of intake
	mg.	%	*mg.*	*mg.*	%	*mg.*	%	*mg.*	*mg.*	%	*mg.*	*mg.*	*mg.*	%	*mg.*	%
V	167	37.1	400	150	0.6	10	— 0.3	0.51	9	— 7	160	0.3	157	0.3	7	— 0.3
C	160	33.9	100	150	2.4	5	— 1.7	0.22	5	— 3	155	0.7	155	1.7	5	— 0.7
J	149	33.7	100	132	1.7	3	— 2.6	0.18	2	— 2	135	— 0.9	146	2.6	14	0.9

Table 6 shows that for some children it was several times as much per day. The 3-year-old girls gained faster than did the older children although they did not store a larger proportion of the nitrogen intake. It must be remembered, however, that the children received these diets for relatively short periods of time. Therefore, the gain during the high protein diet may have been the result of the medium protein diet which was probably better balanced than the diet they had had previous to the study. Nevertheless, these data indicate, as did those of Wang and her associates ('29), that the addition of protein to the diet may produce more rapid gains in weight than a lower protein diet.

These data show that the high protein diet increased the variability of the data during the first three periods and produced greater retentions then than at any other time. After

TABLE 6

Average gain in weight per day

DIET	B	D	E	V	C	J
	gm.	gm.	gm.	gm.	gm.	gm.
Medium protein	1.43	0.95	....	3.81	4.29	2.38
High protein	5.33	4.67	0.00	4.58	8.75	8.75

equilibrium was established it had no effect on the variability of the data or on the correlation with intake, it increased the total amount but not the proportion of total nitrogen excreted, it increased the coefficient of digestibility of the protein because it altered the proportion of nitrogen excreted in the urine and feces, it increased the grams of nitrogen but not the percentage of the intake stored in the body, and it caused a greater gain in weight in the children.

Average values. The average of the retention data per kilogram for all the children for each separate period might rule out individual fluctuations and show general tendencies in the results as compared to the average diet fluctuations. These averages, shown in figure 2, would be possible because the children in each experiment received exactly the same foods per kilogram. It will be seen that on the medium

protein diet the intake and retention variations followed the same general trends, that is, the average retention values tended to fall when the average intake values fell and vice versa. Following the increase in diet nitrogen, the retention values were higher during the first period than at any other time and then they fell slightly and tended to follow the same general course as the intake values. Subject B., in experiment

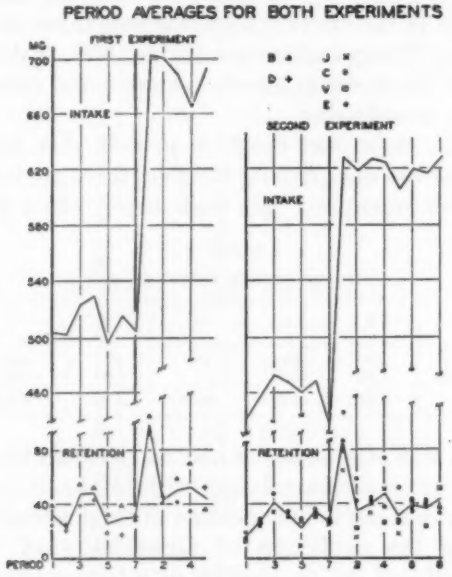

Figure 2

1, seemed to be an exception to this general statement since the average for the high protein diet in that experiment did not seem to follow the same trend. Nevertheless, the graft indicates that the children in the second experiment were in a fair degree of equilibrium during the last 15 and possibly during the last 21 days of the experiment, that they showed the same general trends and that they all tended to react in a similar manner under standard conditions.

SUMMARY

1. Five children received constant diets containing 3 gm. of protein per kilogram. They all excreted the same proportion of the nitrogen intake, but the urine figures fluctuated according to intake variations while the feces values remained constant for each child and were proportional to the dry weight of the feces. The ratio of fecal nitrogen to dry matter consumed remained fairly constant for each child but varied between children according to the character of the stools. For individual children the coefficient of digestibility varied between 86.0 and 92.7%, while the retention values remained more constant but both values fluctuated in proportion to the period by period variation in the diet.

2. Immediately following the change to a 4-gm. protein diet, the excretion values were irregular, but after 9 days they had apparently reached an equilibrium.

3. A comparison of the data following the preliminary period with that on the medium protein diet indicated the effects of protein on the metabolism. The percentage of the intake excreted did not vary, but the total grams increased, practically all of the increase occurring in the urine. The small fecal nitrogen increase was proportional to the increase in dry weight of the feces. The ratio of fecal nitrogen to dry food eaten increased slightly for each child but there was no correlation between the two factors. The coefficient of digestibility was higher for each child. The percentage of the intake nitrogen retained was practically the same, but the total grams increased.

4. The average of the retention and intake values per kilogram for all children for each separate period showed that the children retained nitrogen in relation to the period by period variations except immediately following the change in diet.

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HEAT PRODUCTION AND GASEOUS METABOLISM OF YOUNG MALE CHICKENS

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EIGHT FIGURES

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INTRODUCTION

Modern trends in poultry production have clearly emphasized the necessity of having precise and extensive data available on the energy metabolism of the domestic fowl. Such data are required by the agricultural engineer who is called upon to design the larger poultry plants wherein many thousands of broilers are to be raised, or an equally large number of pullets or hens are to be kept for egg production. However, the ultimate value of such data does not depend solely on their immediate practical utility, because they are also of considerable importance from the standpoint of comparative metabolism.

As has been pointed out by Benedict, Landauer and Fox ('32), who have made a comprehensive review of the literature on the energy metabolism of the domestic fowl, most of the investigations in this field up to the present time have been of a distinctly fragmentary nature. So far as the writers have been able to find, no extensive investigation is recorded in which simultaneous observations on the heat production and gaseous metabolism of the domestic fowl were made in an environment in which the temperature, the humidity, and

the concentration of oxygen and carbon dioxide were kept constant. In the present paper the writers present extensive data so obtained. They present the first curves ever published that show the course of the diurnal rhythm of oxygen consumption of the domestic fowl and how this diurnal rhythm changes with age. They also present some interesting data on the thermogenic effect of casein and gelatin in the domestic fowl.

APPARATUS AND METHODS

The Bureau of Animal Industry's respiration calorimeter at the National Agricultural Research Center, Beltsville, Maryland, was used in obtaining all the data reported in this paper. This respiration calorimeter is an instrument of precision; its construction, the accuracy attainable in its use, and the technic of operation have been discussed in detail by Barott ('37) and briefly by Barott, Byerly and Pringle ('36).

Male Rhode Island Red chickens hatched April 12, 1934, were used. They were separated from the females when 1 day old by the method of visual inspection of the copulatory organ described by Shrader, Burrows and Hammond ('34). After the segregation of the sexes, the males were placed in colony brooder houses on good range and fed a typical all-mash chick diet. The number of male chickens was sufficiently large to make it unnecessary to use the same ones in more than one experiment.

Thirty-five experiments were conducted in which simultaneous measurements of the energy and gaseous metabolism of a suitable number of chicks were made. The first experiment was begun when the chicks were 4 days old and the last when they were 130 days old. Each experiment was of 3 days' duration and when circumstances permitted two experiments were made each week.

The chickens to be used in each of the experiments were removed from the colony house at 8 A.M., brought to the laboratory, weighed, and placed in wire cages of such size as to fit the birds snugly and restrict movements to a minimum. The conditions to prevail during the experiment were then estab-

lished and the measurements of the gaseous metabolism were begun at 10 A.M. and of the heat production at 2 P.M.

At the beginning of the twenty-third hour the chickens were removed from the respiration chamber and weighed immediately. In most of the experiments the birds were then given water and fed a mixture of 98.5% of gelatin, 1.0% of starch and 0.5% of salt, or a similar mixture containing casein in place of the gelatin; but in six of the experiments the birds were given only water. The birds were then weighed a second time and placed again in the respiration chamber.

Measurements of the gaseous metabolism and heat production were begun again at 10 A.M. and 2 P.M., respectively. After the chickens had been under observation for a total period of 72 hours they were removed from the calorimeter, weighed, and returned to their colony houses.

When taken out of the respiration chamber at the beginning of the twenty-third hour, the younger chicks were allowed to eat as much as they would and the quantity of feed consumed was estimated by weighing the feed both before and after the chicks had eaten. After the birds were 6 weeks old a definite quantity of feed was given manually.

The mixture of gelatin, starch and salt contained 10.63% of moisture, 3.20% of ash, 0.11% of ether extractable material, and 15.20% of nitrogen; and the mixture of casein, starch and salt contained 7.46% of moisture, 2.70% of ash, 0.35% of ether extractable material, and 13.73% of nitrogen. Finely powdered salt and starch were added to the gelatin and casein because the resulting mixtures could be fed more easily than the pure gelatin or casein.

The conditions that prevailed in the calorimeter during each of the experiments were: temperature, 90°F.; relative humidity, 60%; oxygen content, 21%; and carbon dioxide content not exceeding 1%. The oxygen consumption of the chickens was determined for each 2-hour period that the chickens were in the calorimeter; carbon dioxide production was measured for the first period of 4 hours and then for each 8-hour period thereafter; direct measurements of heat production

were made during the 8-hour periods when carbon dioxide was being measured but not during the initial 4-hour period.

The respiration calorimeter was checked by alcohol and electric checks made at the beginning and end of the series of experiments. As a result of these checks, as well as of checks previously made, it was found that heat production, oxygen consumption, and carbon dioxide production could be measured with an error of less than $\pm 1\%$. All measuring apparatus was calibrated by the National Bureau of Standards.

PRESENTATION AND DISCUSSION OF THE DATA

The data obtained in this study of the heat production and gaseous metabolism of male Rhode Island Red chickens are too numerous to publish in detail, therefore only suitable summaries are presented.

BASAL METABOLISM

Mitchell and Haines ('27) reported that although the basal metabolism in chickens was often reached after a fasting period of 24 hours, a fast of 48 hours was required before the basal level was reached in all cases. Benedict, Landauer and Fox ('32) also reported that the average fowl must fast 48 hours before its metabolism may be considered as being at the basal level but they believed that if there is not a large quantity of feed in the crop at the beginning of the fast, 36 hours of fasting may suffice to reduce the metabolism to the basal level.

As will be shown later in this paper, the specifications of basal metabolism in the domestic fowl should include the time of day, as well as the number of hours elapsing after the last feed was eaten, because there is a rather pronounced diurnal rhythm of the energy metabolism in this species. The minimum metabolism during any 24-hour period is attained at approximately 8 P.M. and the maximum at approximately 8 A.M. The basal heat production of chickens is usually given on a 24-hour basis, although the measurements are made over a shorter period. If the mid-point of the observational period

is either 2 P.M. or 2 A.M., no serious error will result, but if the observational period is appreciably less than 24 hours and its mid-point is between 2 A.M. and 2 P.M., the estimated heat production for 24 hours will be greater than the true value. Likewise, if the mid-point of such a period is between 2 P.M. and 2 A.M., the estimated value will be less than the true value.

It seems best, therefore, to specify the basal heat production of the chicken as the average number of calories produced per hour during any period of adequate length, when the mid-point of this period is either 2 P.M. or 2 A.M. and occurs a suitable length of time after the last feed was eaten. The writers' data on the basal heat production and gaseous metabolism of male Rhode Island Red chickens are presented in figure 1. The plotted points represent the metabolism observed 66 hours after the last mixed feed had been eaten; but in some cases relatively small quantities of casein or gelatin were fed during the twenty-third hour. In all cases the mid-point of the observational period was 2 A.M. In this figure the metabolism is plotted against age but, inasmuch as the rate of growth is dependent to a considerable extent on the diet fed, the average live weights of the chickens after they had been fasted 66 hours are also plotted against age so that the metabolism for any given live weight, within the range studied, may be read from the plotted curves.

It is of interest to compare the data presented in figure 1 with those published by other investigators. Benedict, Landauer and Fox ('32) did not give the age of their Rhode Island Red chickens, they merely stated that they were hens and cocks; however, most of their birds were heavier than those used by the writers. Nevertheless, it is worth commenting that the oxygen consumption per gram of live weight of their lighter birds was only about two-thirds as great as that observed for the heaviest birds studied by the writers.

Brody ('30) has published some data on the heat production of Rhode Island Red chicks 3 to 56 days old. He computed the heat production from data obtained on oxygen consumption. Using his conversion factor the writers have computed

the oxygen consumption per gram of live weight. The resulting values showed no consistent change with age.

The writers also computed the oxygen consumption per gram of live weight of some White Plymouth Rock chickens studied by Mitchell, Card and Haines ('27). The chickens

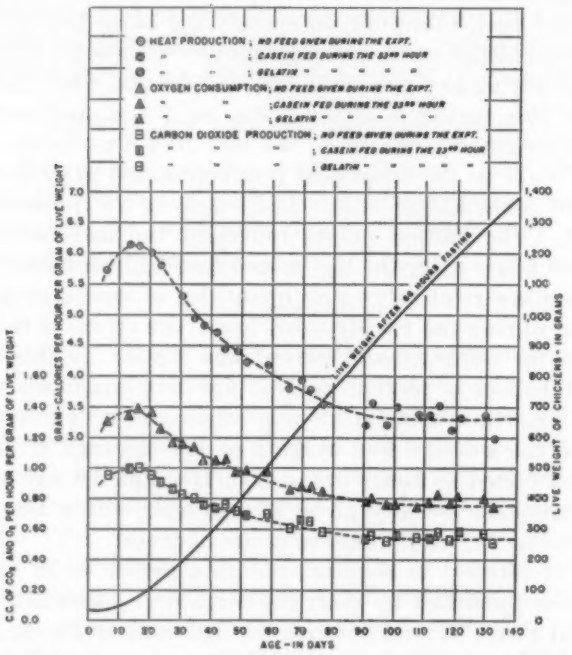
HEAT PRODUCTION; NO FEED GIVEN DURING THE EXPT.
; CASEIN FED DURING THE 23RD HOUR
; GELATIN " " " " "
OXYGEN CONSUMPTION; NO FEED GIVEN DURING THE EXPT.
; CASEIN FED DURING THE 23RD HOUR
; GELATIN " " " " "
CARBON DIOXIDE PRODUCTION; NO FEED GIVEN DURING THE EXPT.
; CASEIN FED DURING THE 23RD HOUR
; GELATIN " " " " "
LIVE WEIGHT AFTER 68 HOURS FASTING
GRAM-CALORIES PER HOUR PER GRAM OF LIVE WEIGHT
C.C. OF CO_2 AND O_2 PER HOUR PER GRAM OF LIVE WEIGHT
LIVE WEIGHT OF CHICKENS - IN GRAMS
AGE - IN DAYS

Fig. 1 Effect of age on the average basal energy and gaseous metabolism of male chickens between 38 and 46 hours after feeding casein or gelatin, and/or between 62 and 70 hours after last mixed feed was consumed.

varied in age from 37 to 355 days. The computed values of the oxygen consumption agreed very closely with those observed by the writers, except in the case of the chickens which were 37 days old.

Mitchell, Card and Haines ('27) also obtained a few data on the oxygen consumption of Rhode Island Red chicks and

from these data computed the heat production. From the supplementary information they supplied on age and live weight, one must conclude that these chicks had grown rather slowly. The average weight of the chicks was only 239 gm. at an average age of 55 days, whereas, after 66 hours of fasting, the chicks used by the writers weighed 465 gm. at that age. However, they report that the average basal heat production of their 239-gm. chicks was 132 kg.-calories per 24 hours, per kilogram of live weight and, as indicated in figure 1, the writers observed an average value of 120 kg.-calories for fasted chicks of the same live weight. On an age basis the agreement is very poor but on a live weight basis it is much better.

An inspection of figure 1 shows that the basal metabolism per gram of live weight increased up to the age of about 15 days—or an average live weight of about 70 gm.—and then decreased to the age of about 100 days—or an average live weight of about 980 gm.—and that after this age was reached the metabolism was fairly constant, at least to the age of 133 days—or an average live weight of 1320 gm. The extent of the decrease in the basal heat production between the ages of 15 and 100 days is indicated by the fact that the basal heat production at the latter age was only about 54% as great as it was at the former age.

MAXIMUM RESTING METABOLISM

For the purpose of factoring into its component parts the total heat production of an animal, it is desirable to have information on the resting metabolism following the ingestion of feed. Accordingly, the data obtained by the writers were examined to determine 1) the relationship between the maximum resting metabolism at 8 A.M.,[1] following the ad libitum ingestion of feed, and the basal metabolism, and 2) the relationship between the average resting metabolism between 8 A.M. and 8 P.M. and the basal metabolism. These relationships are shown in table 1.

[1] The values at 8 A.M. are instantaneous values which were obtained by extrapolation.

It will be seen from the data presented in table 1 that in the very young chick the maximum resting metabolism, after the ad libitum ingestion of feed, is approximately 60% greater than the basal metabolism, whereas when the chick is between 16 and 20 weeks old it is only about 25% greater. However,

TABLE 1

The relationship between the resting energy metabolism, following the ad libitum ingestion of feed, and the basal energy metabolism

AGE	RESTING METABOLISM AT 8 A.M.[1] AS PER CENT OF THE BASAL METABOLISM	AVERAGE METABOLISM BETWEEN 8 A.M. AND 8 P.M. AS PER CENT OF THE BASAL METABOLISM
weeks	%	%
0.5	160	130
1	150	123
2	138	120
3	136	119
4	135	118
5	135	118
6	134	118
7	133	117
8	133	117
9	132	117
10	131	116
11	130	115
12	129	115
13	128	114
14	126	112
15	125	111
16–20	125	109

[1] See footnote 1 on preceding page.

the average metabolism between 8 A.M. and 8 P.M. is only about 30% greater than the basal metabolism in the very young chick and only about 9% greater when the chick is between 16 and 20 weeks old.

R.Q.'S, CO_2 AND O_2 THERMAL QUOTIENTS, AND WATER ELIMINATION

In using the respiratory quotient as an indication of the type of material being metabolized by the chicken, it is necessary to keep in mind that the chief end-product of protein metabolism in this species is uric acid and not urea. Henry, Magee and Reid ('34) appear to have been the first to call

attention to this point. Benedict, Landauer and Fox ('32) and Mitchell and Haines ('27) seem to have ignored the effect on the respiratory quotient of this peculiarity of the protein metabolism in the chicken.

The writers have used the data given by Loewy ('23) on the metabolism of protein and the data of Coulson and Hughes ('30) on the composition of hen urine to estimate the heat production, oxygen consumption, and carbon dioxide and water production resulting from the metabolism of 1 gm. of protein

TABLE 2

Comparison of protein metabolism in the mammal and the bird

CLASS	HEAT PRODUCTION PER GRAM	O_2 CONSUMPTION PER GRAM	CO_2 PRODUCTION PER GRAM	H_2O PRODUCTION PER GRAM	RESPIRATORY QUOTIENT $\left(\frac{CO_2}{O_2}\right)$	CO_2 THERMAL QUOTIENT $\left(\frac{\text{KG.-CALS.}}{\text{GRAMS OF } CO_2}\right)$	O_2 THERMAL QUOTIENT $\left(\frac{\text{KG.-CALS.}}{\text{GRAMS OF } O_2}\right)$
	kg.-cals.	*cc.*	*cc.*	*gm.*			
Mammalia	4.3160	966.3	733.9	0.3960	0.801	2.838	3.124
Aves	3.84	891.1	628.6	0.47	0.705	3.11	3.02

TABLE 3

The heat production per gram and the respiratory and thermal quotients for the metabolism of protein, fat, and starch in the bird

MATERIAL METABOLIZED	HEAT PRODUCTION PER GRAM (KG.-CALS.)	RESPIRATORY QUOTIENT $\left(\frac{CO_2}{O_2}\right)$	CO_2 THERMAL QUOTIENT $\left(\frac{\text{KG.-CALS.}}{\text{GRAMS OF } O_2}\right)$	O_2 THERMAL QUOTIENT $\left(\frac{\text{KG.-CALS.}}{\text{GRAMS OF } CO_2}\right)$
Protein	3.84	0.705	3.110	3.018
Fat	9.4610	0.707	3.373	3.279
Starch	4.1825	1.000	2.568	3.531

in the chicken. These estimates are given in table 2 along with Loewy's values for the mammal. For ready reference, the respiratory and thermal quotients for the metabolism of protein, fat and starch in the bird are given in table 3.

In figure 2 the basal CO_2 production observed between 10 P.M. and 6 A.M. of the third day in the several experiments is plotted against the O_2 consumption; and, for comparison, similar data obtained between 2 P.M. and 10 P.M. of the first day are also plotted. Although the ages of the chickens used

in these experiments ranged from 4 to 133 days and although the gaseous metabolism per gram of live weight varied over a rather wide range, the R.Q.'s showed relatively little variation within each of these two periods. The mean basal R.Q. was 0.719 ± 0.004 [2] and the mean R.Q. during the period between 2 P.M. and 10 P.M. of the first day was 0.788 ± 0.005.

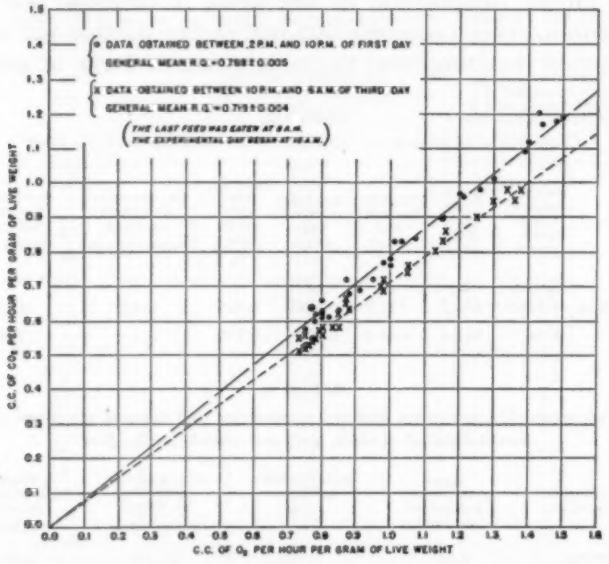
DATA OBTAINED BETWEEN 2 P.M. AND 10 P.M. OF FIRST DAY
GENERAL MEAN R.Q. = 0.788 ± 0.005
DATA OBTAINED BETWEEN 10 P.M. AND 6 A.M. OF THIRD DAY
GENERAL MEAN R.Q. = 0.719 ± 0.004
THE LAST FEED WAS EATEN AT 6 A.M.
THE EXPERIMENTAL DAY BEGAN AT 10 A.M.
C.C. OF CO2 PER HOUR PER GRAM OF LIVE WEIGHT
C.C. OF O2 PER HOUR PER GRAM OF LIVE WEIGHT

Fig. 2 CO_2 production plotted against O_2 consumption for all ages studied.

When the CO_2 production and the O_2 consumption, respectively, were plotted against the heat production, similar results were obtained (figs. 3 and 4). The mean thermal equivalents of CO_2 and O_2 were found to be 6.091 ± 0.038 and 4.377 ± 0.032 kg.-calories per liter, respectively, when the metabolism was at the basal level. During the period between 2 P.M. and 10 P.M. of the first day the mean thermal equivalents of CO_2 and O_2 were 5.909 ± 0.041 and 4.653 ± 0.031 kg.-calories per liter, respectively. The mean CO_2 thermal quo-

[2] Standard errors are used throughout this paper.

tients, when the metabolism was at the basal level and during the period between 2 P.M. and 10 P.M. of the first day, were 3.10 ± 0.02 and 3.01 ± 0.02, respectively.

Brody ('30) adopted the value of 4.825 kg.-cals. per liter (R.Q. = 0.82) as the thermal equivalent of oxygen for normally fed animals about 12 hours after feeding. He also

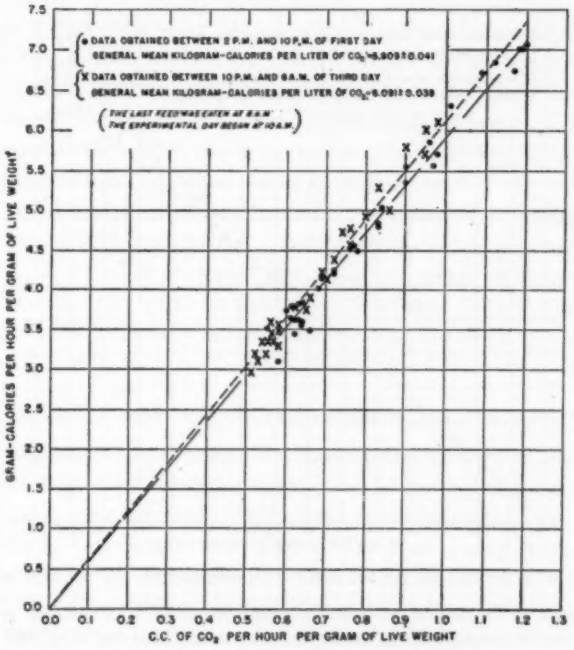
DATA OBTAINED BETWEEN 2 P.M. AND 10 P.M. OF FIRST DAY
DATA OBTAINED BETWEEN 10 P.M. AND 6 A.M. OF THIRD DAY
GRAM-CALORIES PER HOUR PER GRAM OF LIVE WEIGHT
C.C. OF CO₂ PER HOUR PER GRAM OF LIVE WEIGHT

Fig. 3 Heat production plotted against CO_2 production for all ages studied.

concluded that this value may involve an error of 3%. For similar conditions, i.e., 10 hours after feeding, the writers obtained with chickens a mean value of 4.653 ± 0.031 kg.-cals. per liter (R.Q. = 0.788 ± 0.005). This value is only about 3.5% less than the value adopted by Brody.

According to the respiratory and thermal quotients, the chickens were metabolizing mostly protein [3] when their metabolism was at the basal level. However, during the period between 2 P.M. and 10 P.M. of the first day, when the chickens were still in the absorptive stage, they were metabolizing an appreciable quantity of carbohydrate.

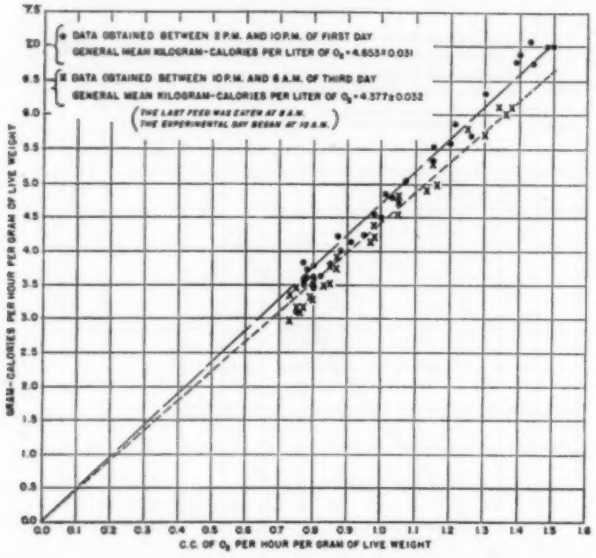
DATA OBTAINED BETWEEN 2 P.M. AND 10 P.M. OF FIRST DAY
GENERAL MEAN KILOGRAM-CALORIES PER LITER OF O2 = 4.855±0.031
DATA OBTAINED BETWEEN 10 P.M. AND 6 A.M. OF THIRD DAY
GENERAL MEAN KILOGRAM-CALORIES PER LITER OF O2 = 4.377±0.032
(THE LAST FEED WAS EATEN AT 6 A.M.
THE EXPERIMENTAL DAY BEGAN AT 10 A.M.)
GRAM-CALORIES PER HOUR PER GRAM OF LIVE WEIGHT
C.C. OF O2 PER HOUR PER GRAM OF LIVE WEIGHT

Fig. 4 Heat production plotted against O_2 consumption for all ages studied.

[3] Inasmuch as the writers made direct measurements of both the heat production and gaseous metabolism, they were able to estimate from the R.Q. and the CO_2 and O_2 thermal quotients the relative quantities of carbohydrate, fat, and protein being metabolized. One method of estimation was as follows: If x, y and z represent, respectively, the percentages of carbohydrate, fat, and protein being metabolized, the following equations may be set up and readily solved:

$$1.000\ x + 0.707\ y + 0.705\ z = 100 \times \text{the observed R.Q.}$$
$$2.568\ x + 3.373\ y + 3.110\ z = 100 \times \text{the observed } CO_2 \text{ thermal quotient.}$$
$$3.531\ x + 3.279\ y + 3.018\ z = 100 \times \text{the observed } O_2 \text{ thermal quotient.}$$

The average numerical values of the R.Q., CO_2 thermal quotient, and O_2 thermal quotient of the chickens when their metabolism was at the basal level were 0.719, 3.100 and 3.063, respectively. If the equations given above are solved after these values are inserted in the proper places, it will be found that approximately 5% of carbohydrate, 10% of fat and 85% of protein were being metabolized.

Benedict, Landauer and Fox ('32) referred to the 'fat quotient' of the basal state and implied that birds in the basal state metabolize chiefly fat. Also, Mitchell and Haines ('27) concluded, on the basis of the respiratory quotients which they obtained, that "the metabolism of the chicken in the period from the forty-eighth to the seventieth hours after feeding is almost entirely at the expense of fat." That either Benedict, Landauer and Fox's or Mitchell and Haines' chickens, when in the basal state, metabolized very much fat is questionable. The writers observed almost exactly the same R.Q. for birds in the basal state as did Mitchell and Haines but the thermal quotients observed by the writers clearly indicate that only a small quantity of fat was being metabolized.[4]

The total water elimination, when expressed as milligrams per gram of live weight, was found to be fairly constant for all ages. It was 2.8 ± 0.1 mg. per hour per gram of live weight when the chickens were at the basal level and 3.1 ± 0.1 mg. per hour per gram during the 8-hour period between 2 P.M. and 10 P.M. of the first day of the experiments.

DIURNAL RHYTHM OF THE ENERGY METABOLISM

The course of the diurnal rhythm of the energy metabolism of the chicken, as it changes from hour to hour during a period of 24 hours, has not previously been determined.

Benedict, Landauer and Fox ('32) made day and night measurements of the gaseous metabolism of chickens and found that the values obtained during the day were approximately 10% greater than those obtained during the night. Brody ('30) made some early morning and late evening observations on the oxygen consumption of fasting chickens. His results were somewhat erratic since some of the evening values were greater than the morning values. However, in general, he obtained the larger values during the morning.

Mitchell, Card and Haines ('27) also observed higher values for the oxygen consumption during the day than they did at night. They at first thought that this difference in metabolism was a diurnal rhythm phenomenon but finally concluded

[4] See footnote 3 on preceding page.

that a more probable explanation was that the observed differences were due to muscular activity. In their youngest birds the difference between morning and afternoon values averaged 21% and in their oldest birds about 12%, but to them

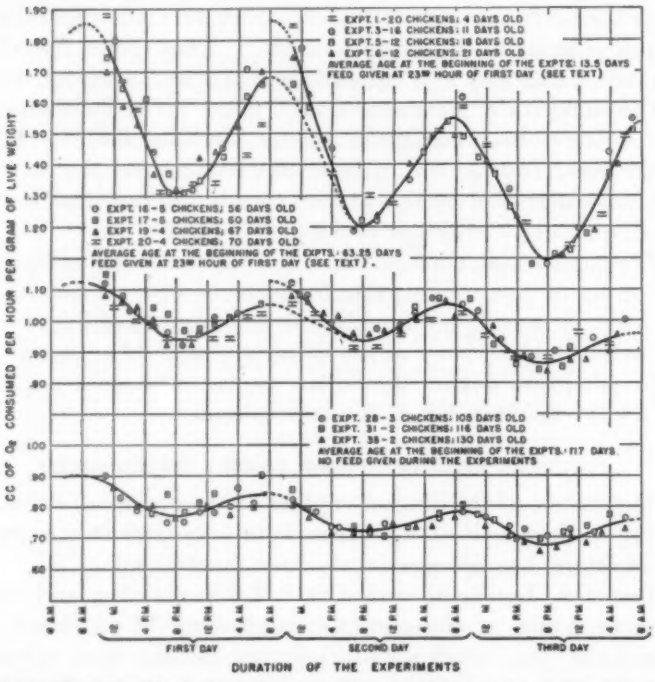
= EXPT. 1-20 CHICKENS: 4 DAYS OLD
○ EXPT. 3-16 CHICKENS: 11 DAYS OLD
□ EXPT. 5-12 CHICKENS: 18 DAYS OLD
△ EXPT. 6-12 CHICKENS: 21 DAYS OLD
AVERAGE AGE AT THE BEGINNING OF THE EXPTS: 13.5 DAYS
FEED GIVEN AT 23RD HOUR OF FIRST DAY (SEE TEXT)
○ EXPT. 16-5 CHICKENS: 56 DAYS OLD
□ EXPT. 17-6 CHICKENS: 60 DAYS OLD
△ EXPT. 19-4 CHICKENS: 67 DAYS OLD
= EXPT. 20-4 CHICKENS: 70 DAYS OLD
AVERAGE AGE AT THE BEGINNING OF THE EXPTS.: 63.25 DAYS
FEED GIVEN AT 23RD HOUR OF FIRST DAY (SEE TEXT).
○ EXPT. 28-3 CHICKENS: 105 DAYS OLD
□ EXPT. 31-2 CHICKENS: 116 DAYS OLD
△ EXPT. 38-2 CHICKENS: 130 DAYS OLD
AVERAGE AGE AT THE BEGINNING OF THE EXPTS.: 117 DAYS.
NO FEED GIVEN DURING THE EXPERIMENTS
CC OF O_2 CONSUMED PER HOUR PER GRAM OF LIVE WEIGHT
1.90
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
.90
.80
.70
.60
FIRST DAY
SECOND DAY
THIRD DAY
DURATION OF THE EXPERIMENTS

Fig. 5 The diurnal rhythm of the energy metabolism, as measured by the oxygen consumption, of fasting male chickens at different ages.

these differences seemed to be too large to be ascribable to diurnal rhythm.

The data on diurnal rhythm reported in this paper are for oxygen consumption, inasmuch as the carbon dioxide and heat production were not measured at sufficiently frequent intervals to show the relatively smooth course of the change in metabolism.

That there is a very definite rhythm in the oxygen consumption of chickens is clearly shown in figure 5, in which data from several of the writers' experiments have been plotted. The marked break in each of the first two curves at the end of the first 24 hours was caused by the protein that was fed during the twenty-third hour.

The effect of age on the diurnal rhythm is indicated in figure 5 but is shown more clearly in figure 6, in which the average amplitude of the rhythm for several consecutive experiments is plotted against the average age of the chicks. At the average age of 1 week the amplitude is about 12% of the oxygen consumption at 8 A.M. This means that an average maximum difference of about 24% was observed between the

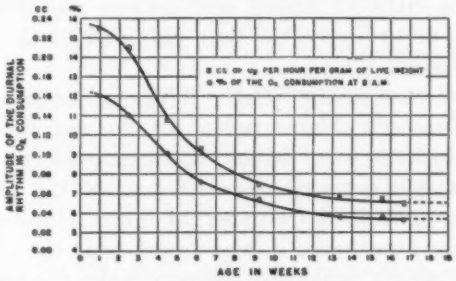

Fig. 6 Effect of age on the amplitude of the diurnal rhythm of the oxygen consumption per gram of live weight of male chickens.

oxygen consumption at 8 A.M. and at 8 P.M. After the age of 14 weeks was reached the amplitude of the diurnal rhythm tended to remain constant at a value of about 5.7% of the oxygen consumption at 8 A.M. This is equivalent to a difference of about 11.4% between the 8 A.M. and 8 P.M. values for oxygen consumption. These differences between the morning and evening results are of the same order of magnitude as those reported by Mitchell, Card and Haines ('27).

In view of the fact that the energy metabolism of the chicken exhibits a definite diurnal rhythm of considerable magnitude it is clear that specifications of the basal metabolism in the domestic fowl should include the time of day, as well as the number of hours elapsing after the last feed was eaten.

THE THERMOGENIC EFFECTS OF CASEIN AND GELATIN

The various theories of thermogenic action or, as it is more commonly called, specific dynamic action (SDA), have recently been reviewed by Borsook ('36), Wilhelmj ('35), Brody ('34) and Brody and Proctor ('33). In view of the fact that protein metabolism follows different courses in the mammal and the bird, comparative studies of SDA in these two classes should be of value in checking some of the theories.

Only a few studies have been reported on the thermogenic effect of feeding stuffs in the chicken and these have been reviewed by Mitchell and Haines ('27). In these studies, and in those of Mitchell and Haines ('27), corn was the only feeding stuff used. There appears to be no record of experiments in which the SDA of casein and gelatin was determined in the chicken; accordingly, the data obtained by the writers are of some interest.

Method of measuring SDA. When the total oxygen consumption, for each 2-hour period of the six experiments in which the chickens were fasted, was expressed as per cent of the total oxygen consumption at 8 A.M. of the first day and then plotted against the number of hours elapsing after the last feed was consumed, it was found that the resulting six curves very nearly coincided. Accordingly, the values for the total oxygen consumption for each of the corresponding periods during the last 48 hours of the fasting experiments were expressed as per cent of the 8 A.M. value, averaged, and then plotted against the number of hours elapsing after the last feed was consumed. Since no feed was fed during the first 22 hours in any of the experiments, the appropriate data from the last eighteen experiments were used to complete the curve for this period. The resulting composite curve is shown in figure 7. This curve, therefore, shows how the total oxygen consumption of fasting chickens, when expressed as per cent of the total oxygen consumption at the beginning of the fast, changed as the fast continued.

By using the data plotted in figure 7 it was possible to compute for those experiments, in which casein or gelatin was fed

during the twenty-third hour, what the oxygen consumption would have been if the casein or gelatin had not been fed. The difference between the total quantity of oxygen actually consumed and that which would have been consumed, if no casein or gelatin had been fed, was considered as a measure of the thermogenic effect of the protein fed.

The procedure followed in estimating the SDA of the casein and gelatin is illustrated in figure 8. The upper curve in each of the two pairs of curves represents the observed oxygen consumption and the lower curve represents the oxygen consumption which would have been observed if no protein had

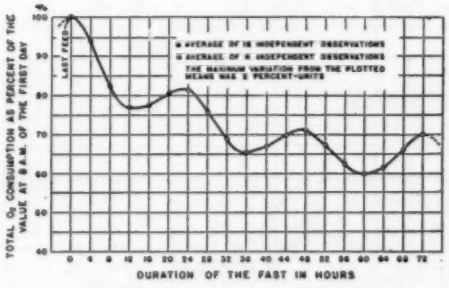

Fig. 7 Change in the total energy metabolism (as measured by the oxygen consumption), during a 3-day fast, of male chickens which were between 63 and 131 days old at the beginning of the fast.

been fed. For the first 24 hours, in the case of the lower curve of each pair, both the observed and computed oxygen consumption have been plotted to show the agreement between the two.

The rate of decrease of the oxygen consumption during the last 48 hours was always greater for the birds which were fed during the twenty-third hour than it was for the birds which were not fed. Accordingly, it was found that the curve representing the observed oxygen consumption and the curve representing the oxygen consumption which would have been observed, if no protein had been fed, eventually coincided. In the case of the younger birds the two curves coincided about

36 hours after feeding casein and about 48 hours after feeding gelatin. As the age of the birds increased the length of time required for the curves to coincide tended to increase, but it was always about two-thirds as great when casein was fed as when gelatin was fed.

After the observed and computed oxygen consumption had been plotted, as shown in figure 8, the area between the two

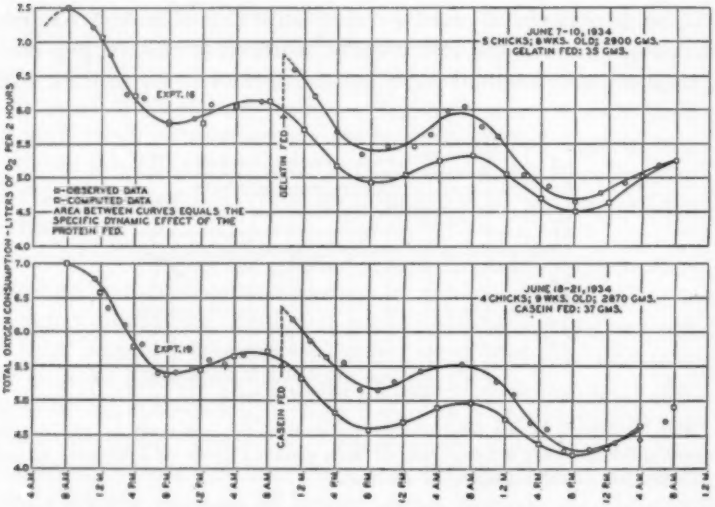
JUNE 7-10, 1934
5 CHICKS; 8 WKS. OLD; 2900 GMS.
GELATIN FED: 35 GMS.
EXPT. 16
O-OBSERVED DATA
O-COMPUTED DATA
AREA BETWEEN CURVES EQUALS THE SPECIFIC DYNAMIC EFFECT OF THE PROTEIN FED.
GELATIN FED
JUNE 18-21, 1934
4 CHICKS; 9 WKS. OLD; 2870 GMS.
CASEIN FED: 37 GMS.
EXPT. 19
CASEIN FED
TOTAL OXYGEN CONSUMPTION - LITERS OF O2 PER 2 HOURS

Fig. 8 Oxygen consumption during experiments 16 and 19, as observed for male chickens fed gelatin and casein, respectively, and as computed for fasting chickens of the same age.

resulting curves was carefully measured with a polar planimeter. The resulting estimates of the SDA of casein and gelatin in sixteen of the experiments are presented in table 4.

An examination of the data in table 4 shows that both the average initial increase in oxygen consumption and the average total increase per gram of nitrogen were greater for casein than for gelatin. If one uses 4.52 kg.-cal. per liter [5]

[5] An average of the thermal equivalents which were observed a) 10 hours after the last mixed feed was fed and b) when the metabolism was at the basal level.

TABLE 4

The specific dynamic action (SDA) of the two proteins, casein and gelatin, when fed to male chickens of different ages between 40 and 128 days, as measured by the oxygen consumption[1]

EXPERIMENT NO.	AGE OF CHICKS	NUMBER OF CHICKS	TOTAL WEIGHT OF THE CHICKS WHEN THE PROTEIN WAS FED	QUANTITY OF NITROGEN IN THE PROTEIN FED			TOTAL O_2 CONSUMPTION DURING THE 2-HOUR PERIOD BEFORE THE PROTEIN WAS FED	INCREASE IN O_2 CONSUMPTION DURING FIRST 2-HOUR PERIOD AFTER PROTEIN WAS FED			SDA	
				Total	Per chick	Per kilogram of live weight		Total	Per gram of nitrogen	As per cent of O_2 consumption before feeding	Total increase in O_2 consumption	Increase in O_2 consumption per gram of nitrogen
							Casein					
	days		*kg.*	*gm.*	*gm.*	*gm.*	*liters*	*liters*	*liters*	%	*liters*	*liters*
11	40	6	2.08	4.12	0.69	1.98	5.5	1.0	0.24	18	8.33	2.02
14	50	6	3.04	5.49	0.92	1.81	6.8	1.2	0.22	18	12.54	2.28
17	61	5	2.75	5.49	1.10	2.00	5.9	1.0	0.18	17	12.20	2.22
19	68	4	2.78	5.08	1.27	1.83	5.7	0.8	0.16	14	10.28	2.02
23	89	4	5.00	6.87	1.72	1.37	8.2	1.1	0.16	13	14.33	2.09
26	99	4	4.32	8.92	2.23	206	7.8	1.4	0.16	18	19.24	2.16
29	110	3	3.56	10.30	3.43	2.89	5.8	1.3	0.13	22	21.85	2.12
32	120	2	3.24	8.24	4.12	2.54	5.2	1.1	0.13	21	18.64	2.26
Mean	...	..	...	...	...	...	..	1.1	...	18	....	2.15
							Gelatin					
12	43	6	2.53	4.56	0.76	1.80	6.1	0.8	0.18	13	8.62	1.89
13	47	6	2.73	4.56	0.76	1.67	6.3	0.8	0.18	13	8.70	1.91
16	57	5	2.90	5.32	1.06	1.83	6.1	0.8	0.15	13	10.33	1.94
20	71	4	3.26	6.08	1.32	1.87	6.5	0.9	0.15	14	12.18	2.00
24	92	4	4.00	9.12	2.28	2.28	6.8	0.9	0.10	13	18.46	2.02
27	103	3	3.17	9.12	3.04	2.88	5.5	0.9	0.10	16	17.21	1.89
30	113	2	2.52	9.88	4.94	3.92	4.1	0.8	0.08	20	18.15	1.84
34	128	2	2.60	9.12	4.56	3.51	3.9	0.8	0.09	21	17.28	1.89
Mean	...	..	...	...	...	...	..	0.8	...	15	....	1.92

[1] In the first ten experiments, the ad libitum consumption of casein and gelatin was small and irregular and so the data obtained on SDA in these experiments are not given. In the subsequent experiments with casein and gelatin the chickens were forcibly fed. In experiments 18, 21, 25, 28, 31 and 35 the chickens were fasted for 3 days and, hence, no casein or gelatin was fed. It was not possible to complete experiments 15, 22 and 33.

as the average thermal equivalent of oxygen after casein or gelatin was fed, it is found that the average SDA of casein was 9.7 kg.-cal. per gram of nitrogen and of gelatin 8.7 kg.-cal. per gram of nitrogen. Apparently the SDA of casein and gelatin was not affected by age, although the duration of the SDA was longer in the older chickens than it was in the younger ones.

Attention is directed to the fact that the SDA, its initial intensity, and its duration are not the same for casein and gelatin. The duration of the SDA was not so long for casein as it was for gelatin, but the initial oxygen consumption during the first 2 hours reached higher levels after casein was ingested than it did after gelatin was ingested. The difference in SDA between casein and gelatin is fairly large and, statistically, it is highly significant.

Although no determinations of the nitrogen excretion of the chickens were made, it is apparent that, in effect, the method of computing SDA used by the writers was essentially the same as that recommended by Borsook ('36), inasmuch as in both methods the SDA may be expressed as the excess calories of heat produced per gram of nitrogen in the protein fed. Accordingly, it must be concluded that, in general, the SDA of casein and gelatin in the chicken is not so great as it is in man or the dog, because, according to Borsook's ('36) calculations, the SDA of casein in man was between 14 and 21 kg.-cal. per gram in Gigon's experiments and the SDA of gelatin in the dog was between 12 and 15 kg.-cal. per gram in Rapport and Beard's experiments.

SUMMARY AND CONCLUSIONS

Thirty-five experiments, each of 72 hours duration, were made in which energy and gaseous metabolism of male Rhode Island Red chickens between the ages of 4 and 133 days was measured. The conditions prevailing in the calorimeter during each experiment were: temperature, 90°F.; relative humidity, 60%; O_2 content, 21%; and CO_2 content, not exceeding 1%. The oxygen consumed during periods of 2 hours and the

carbon dioxide and heat produced during periods of 8 hours were determined. In some of the experiments either casein or gelatin was fed during the twenty-third hour in order to study the thermogenic effect of these two proteins.

Data on basal metabolism, maximum resting metabolism, R.Q.'s, CO_2 and O_2 thermal quotients and equivalents, total water elimination, diurnal rhythm, and the thermogenic effects of casein and gelatin are given. Curves are presented that show the course of the diurnal rhythm of oxygen consumption in the domestic fowl, and how the amplitude of this diurnal rhythm changes with age.

It is concluded that:

1. The basal energy metabolism per gram of live weight, after 66 hours of fasting, is greatest in the male Rhode Island Red chicken when it is about 15 days old or when the weight is about 70 gm. Thereafter the basal energy metabolism decreases at a rapid rate until it becomes relatively constant at an age of about 100 days, or an average weight of about 980 gm.

2. In the very young chick the maximum resting metabolism after the ingestion of feed is about 60% greater than the basal metabolism, whereas between the ages of 16 and 20 weeks it is only about 25% greater.

3. The basal R.Q. is very nearly the same for all ages between 4 and 133 days; it is 0.719 ± 0.004.

4. The total water elimination per gram of live weight was fairly constant at all ages studied. It was 2.8 ± 0.1 mg. per hour per gram when the chickens were at the basal level and 3.1 ± 0.1 mg. per hour per gram during the 8-hour period between 2 P.M. and 10 P.M. of the first day of the experiment.

5. The thermal equivalent of oxygen in the chicken is 4.653 ± 0.031 kg.-calories per liter about 10 hours after the last ingestion of feed; and 4.377 ± 0.039 kg.-calories per liter when the fasting metabolism is at the basal level.

6. The maximum energy metabolism occurs at about 8 A.M. and the minimum at about 8 P.M.

7. The amplitude of the diurnal rhythm of the energy metabolism is greatest in the very young chick and decreases rapidly with age.

8. In the chicken the SDA of casein is about 9.7 kg.-calories per gram of nitrogen and that of gelatin is about 8.7 kg.-calories per gram of nitrogen. The difference is statistically significant.

9. The SDA of casein and gelatin in the growing chicken is apparently not affected by age but the duration of the SDA is longer in the older chickens than it is in the younger ones, and longer for gelatin than it is for casein.

10. The SDA of casein and gelatin in the chicken is not so great as it is in man or the dog.

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THE TRACE ELEMENT CONTENT OF THE NEWBORN RAT (AS DETERMINED SPECTROGRAPHICALLY)

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So far as could be determined by a search of the literature, observations of trace elements in the normal newborn rat have been reported for only a few elements, namely; aluminum (Myers and Mull, '28), cobalt (Stare and Elvehjem, '33), copper (Lindow, Peterson and Steenbock, '29), manganese (Skinner, Peterson and Steenbock, '31; Orent and McCollum, '31), and zinc (Thompson, Marsh and Drinker, '27; Bertrand and Beauzemont, '30; Newell and McCollum, '33).

Lack of suitable methods prior to the extensive use of spectroscopy made difficult the determination of more than one trace element at a time in the ash obtained from an individual newborn rat.

This paper reports the spectrographic estimation of trace elements in the ash of the newborn rat.

EXPERIMENTAL

The rats (Mus norvegicus) used in this study were propagated from a foundation colony secured from Dr. L. S. Palmer, University of Minnesota in 1936. The stock diet consisted of a commercial fox feed [1] which was fed 'ad libitum,' supplemented daily by 20 cc. of fresh whole milk, and lettuce twice weekly. Ordinary distilled water was available to the animals at all times.

[1] Champion 100% fox feed. Ingredients, dehydrated meat, skim milk powder, oat meal, yellow corn meal, bran, red dog flour, second clear flour, pure wheat germ, steamed bonemeal, calcium carbonate, iodized salt and cod liver oil. Analysis, moisture 6.7%, protein 31.0%, fat 8.0%, ash 7.0%, fiber 4.8%, and nitrogen free extract 42.5%.

Prior to parturition the mothers were placed in individual cages provided with wood shavings. The young were taken as soon as possible after birth, association with the mother never exceeding 1 day.

The newborn were brushed free from any wood shavings, rinsed with redistilled water, and placed in platinum dishes for drying at 100°C. All glassware and platinum used in the preparation of the ash was leached with hot 1:1 hydrochloric acid and rinsed several times with water, triple-distilled through a silica still. After drying, the animals were ashed in a muffle furnace at a temperature not exceeding 450°C. for 24 to 48 hours. The ash was then homogenized in an agate mortar and analyzed spectrographically.

Spectrographic technic

The procedure was designed to permit an estimation of the proportions of certain elements present in magnitudes detectable by spectrography on the original sample.

A Littrow spectrograph with a linear dispersion of about 30 inches between 2250 Å and 5500Å was used. Two prisms were used with this instrument, a glass prism for lines of wave length greater than 3800 Å and a quartz prism for shorter wave lengths.

A small aliquot of the homogenized ash was volatilized in a 220 volt arc using a current of 9 to 10 amperes. Specially purified graphite was used as electrodes. Repeated spectra of the graphite electrodes were made to insure a control of electrode impurities. In taking the spectrum of the sample, the arc was maintained until the sample was completely volatilized. Incomplete volatilization would permit fractionation, involving a possible retention of the higher boiling elements in the residue. This might vitiate estimates of the amounts of the elements present.

A mixture of the twenty-four elements included in the analysis was used as a wave length standard, by means of which the lines in the spectra of the sample were identified.

The estimations were made by the comparison method used by Nitchie ('29). Standard powders containing known per-

centages of the various elements were spectrographed in juxtaposition with spectrograms of the ash samples. The percentages of the elements present were then estimated by comparing visually the intensities of spectral lines in the sample with corresponding lines in the standards. Duplicate determinations were made on each sample.

The data thus obtained are not intended as precision determinations, but are indicative of the 'order of magnitude' of the proportions of the elements present. To avoid misunderstanding as to precision, and also to retain a legitimate basis for comparison, the data are presented in range form. (For example, 0.001–0.005 recorded in the table should be read: The amount of the element in the sample lies between 0.001 and 0.005 mg.)

The approximate sensitivity of the method is: between 0.0001 and 0.001% for chromium, cobalt, copper, nickel and silver; between 0.001 and 0.01% for aluminum, barium, beryllium, manganese, molybdenum, lead, strontium, tin, titanium, vanadium and zinc; between 0.01 and 0.1% for antimony, bismuth, boron, cadmium, lanthanum, thallium, yttrium and zirconium.

In view of the varying sensitivities listed above, the term 'trace' used in the table has a varying significance. In all cases, the term 'trace' corresponds to the lower limit of sensitivity for the element involved. Thus a trace of chromium would signify about 0.001%, whereas a trace of boron would signify about 0.01%.

RESULTS

The results of the spectrographic analyses of fourteen newborn rats from six different litters (not more than three of which were from any one litter) and of the stock diet of the mothers are given in tables 1 and 2. The amount of the element present is expressed in milligrams per rat and in p.p.m. of the feed. The live weights of the animals, the ash weights, the percentage of moisture and the percentage of ash on the fresh basis also are recorded.

TABLE 1

Spectrographic estimation of aluminum, barium, chromium, copper, lead and manganese in newborn rats and in the stock feed of the mothers. The values are expressed in milligrams per rat and in p.p.m. for the stock feed on the fresh basis[1]

RAT NO.	WEIGHT	ASH	MOISTURE	ASH (WET BASIS)	ALUMINUM	BARIUM	CHROMIUM	COPPER	LEAD	MANGANESE
	gm.	gm.	%	%						
2227	5.727	0.096	85.2	1.67	0.0008–0.003	0.001	0.001	0.008–0.03	0.0008–0.003	0.0008–0.003
2276	5.837	0.104	85.2	1.78	0.001	0.001	0.008–0.03	0.01–0.05	Tr	0.0003–0.008
2277	5.230	0.094	83.9	1.79	0.0009	0.0009	N.D.	0.007–0.03	0.0009–0.005	0.0009–0.005
2278	5.400	0.092	84.1	1.71	0.0009	0.0009	N.D.	0.007–0.03	0.0009	0.0007–0.003
2279	5.340	0.096	85.3	1.79	0.001	0.001	N.D.	0.03–0.08	0.001	0.001
2280	5.369	0.091	85.1	1.69	0.0007–0.003	0.0009	Tr	0.007–0.03	Tr	0.0007–0.003
2281	4.858	0.094	85.0	1.94	0.0008–0.003	0.0009	N.D.	0.008–0.03	0.0009	0.0009
2282	5.741	0.096	83.2	1.68	Tr	0.001	Tr	0.008–0.03	0.001	0.0008–0.003
2283	5.757	0.098	83.5	1.70	.0008–0.003	0.001	0.001	0.05–0.1	0.0008–0.003	0.001
2284	5.020	0.094	85.4	1.87	0.0009	Tr	N.D.	0.009–0.05	0.0009	0.0009
2285	6.126	0.103	82.6	1.67	0.001	0.001	N.D.	0.03–0.08	0.001	0.001
2286	5.282	0.093	85.9	1.76	0.0007–0.003	0.0009	N.D.	0.007–0.03	†	0.0009
2287	5.522	0.095	85.5	1.72	0.001	0.001	N.D.	0.009–0.05	†	0.0008–0.003
2288	5.696	0.099	84.9	1.73	0.0008–0.003	0.001	Tr	0.008–0.03	N.D.	0.001
Stock feed	56.945	3.84	7.85	6.74	34.0–67.0	7.0–34.0	Tr	5.0–20.0	0.5–2.0	20.0–54.0

[1] The approximate sensitivity of the method is: for chromium, cobalt, copper, nickel and silver, between 0.0001 and 0.001%; for aluminum, barium, beryllium, manganese, molybdenum, lead, strontium, tin, titanium, vanadium and zinc, between 0.001 and 0.01%; for antimony, bismuth, boron, cadmium, lanthanum, thallium, yttrium and zirconium, between 0.01 and 0.1%. 'N.D.' denotes 'not detected.' 'Tr' denotes 'trace.' '†' denotes trace, but not positive identity.

TABLE 2

Spectrographic estimation of molybdenum, nickel, silver, strontium, tin and zinc in newborn rats and in the stock feed of the mothers. The values are expressed in milligrams per rat and in p.p.m. for the stock feed on the fresh basis[1]

RAT NO.	WEIGHT	ASH	MOISTURE	ASH (WET BASIS)	MOLYBDENUM	NICKEL	SILVER	STRONTIUM	TIN	ZINC
	gm.	*gm.*	%	%						
2227	5.727	0.096	85.2	1.67	Tr	0.001	0.001	0.001	0.001	0.05–0.1
2276	5.837	0.104	85.2	1.78	N.D.	0.008–0.03	Tr	0.001	Tr	0.08–0.3
2277	5.230	0.094	83.9	1.79	N.D.	N.D.	Tr	0.0009	0.0009	0.05–0.09
2278	5.400	0.092	84.1	1.71	N.D.	N.D.	Tr	0.0009	0.0008–0.003	0.07–0.3
2279	5.340	0.096	85.3	1.79	N.D.	N.D.	N.D.	0.001	0.001	0.08–0.3
2280	5.369	0.091	85.1	1.69	N.D.	N.D.	Tr	0.0009	0.0008–0.003	0.07–0.3
2281	4.858	0.094	85.0	1.94	N.D.	N.D.	Tr	0.0009	0.0009	0.07–0.1
2282	5.741	0.096	83.2	1.68	N.D.	N.D.	Tr	0.001	0.001	0.05–0.09
2283	5.757	0.098	83.5	1.70	N.D.	0.001–0.005	Tr	0.001	0.0008–0.003	0.08–0.3
2284	5.020	0.094	85.4	1.87	N.D.	N.D.	N.D.	Tr	0.0009	0.08–0.3
2285	6.126	0.103	82.6	1.67	N.D.	N.D.	Tr	0.001	0.0008–0.003	0.08–0.3
2286	5.282	0.093	85.9	1.76	N.D.	N.D.	Tr	0.0009	0.0009	0.08–0.3
2287	5.522	0.095	85.5	1.72	N.D.	N.D.	N.D.	0.001	Tr	0.08–0.3
2288	5.696	0.099	84.9	1.73	N.D.	N.D.	N.D.	0.001	Tr	0.08–0.3
Stock feed	56.945	3.84	7.85	6.74	0.5–2.0	2.0–5.0	N.D.	0.7	Tr	67.0

[1] The approximate sensitivity of the method is: for chromium, cobalt, copper, nickel and silver, between 0.0001 and 0.001%; for aluminum, barium, beryllium, manganese, molybdenum, lead, strontium, tin, titanium, vanadium and zinc, between 0.001 and 0.01%; for antimony, bismuth, boron, cadmium, lanthanum, thallium, yttrium and zirconium, between 0.01 and 0.1%. 'N.D.' denotes 'not detected.' 'Tr' denotes 'trace.'

Aluminum, barium, copper, manganese, strontium, tin and zinc were present in all of the rat ashes. Lead was detected in eleven of the fourteen rat ashes; silver in ten; chromium in six; nickel in three; and molybdenum in one. The following elements were detected in the stock feed; aluminum, barium, chromium, copper, lead, mangenese, molybdenum, nickel, strontium, tin, titanium and zinc. Antimony, beryllium, bismuth, boron, cadmium, cobalt, lanthanum, thallium, vanadium, yttrium, and zirconium were not detected in any of the ash samples. Titanium was not detected in the ash of the rats but a trace appeared in the ash of the feed.

DISCUSSION

Certain elements were detected in all of the ash samples, hence, the results for these elements are at least consistent. Other elements were detected irregularly in the animal ashes which might indicate that this lack of consistency is due either to contamination of the ash in some manner, or to differences in placental transmission.

In general, as pointed out previously, the small quantity of ash obtained from a normal newborn rat does not permit an extensive chemical analyses of the ash. Consequently, the data in the literature consist primarily of the determination of only one element in a given sample of ash. In the case of those elements which have been determined previously, the results, obtained in this study are in reasonable agreement with the published findings of other investigators.

Zinc. The value of the zinc content was the highest attained for any of the trace elements present in the ash of the normal newborn rat. Thompson, Marsh and Drinker ('27) reported a zinc content of 0.038 mg. per gram of rat; and Newell and McCollum ('33), using a spectrographic method, found 0.37 mg. per animal. Bertrand and Beauzemont ('30) reported 37.51 mg. of zinc per 100 gm. of dry total body tissue (1-day-old rats), which appear to be somewhat higher than the above values and those reported in this paper.

Copper. Copper was found in the next highest amount. Lindow, Steenbock and Elvehjem ('29) reported 0.0089 to 0.0134 mg. of copper per animal in their normal newborn rats.

Manganese. By means of spectrographic technic, Orent and McCollum ('31) found 0.1 to 0.5 p.p.m. of manganese in normal newborn rats, while Skinner, Peterson and Steenbock ('31) reported 0.0013 to 0.0019 mg. of manganese per rat.

Cobalt. Cobalt could not be detected by Stare and Elvehjem ('33) in newborn rats whose mothers were on an experimental diet free from cobalt. Neither was cobalt detected in the ashes analyzed in this study.

Aluminum. The values for aluminum found in this investigation, however, are much lower than those reported by Myers and Mull ('28), who found 0.33 mg. per 100 gm. of fetal young, and 0.20 mg. per 100 gm. of young (?) animal. The values are approximately 0.01 to 0.02 mg. per rat when computed on the basis of an approximate weight of 5 gm. for a newborn rat.

The following elements: barium, chromium, lead, molybdenum, nickel, silver, strontium, and tin were found in smaller quantities (traces). A search of the literature failed to disclose any of these as having been reported previously in the normal newborn rat.

The fact that twelve of the twenty-four elements studied could not be detected in the animal ashes does not mean that these were not present. They might be present in amounts smaller than the sensitivity of the method in use.

The common presence of aluminum, barium, copper, lead, manganese, strontium, tin and zinc in these ashes suggests that these elements are probably transmitted through the placenta.

Other elements detected irregularly in the ash samples, might also be involved in placental transmission; the evidence, however, is insufficient to warrant a definite conclusion.

SUMMARY

Spectrographic estimation of trace elements in normal newborn rats showed aluminum, barium, copper, manganese, strontium, tin and zinc, to be present in all of the animals. Lead and silver were detected in more than half the rat ash samples, Antimony, beryllium, bismuth, boron, cadmium, cobalt, lanthanum, thallium, titanium, vanadium, yttrium and zirconium were not detected in any of the rat samples.

It is suggested that the following elements might be transmitted from mother to young—aluminum, barium, copper, lead, manganese, strontium, tin and zinc.

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THE CHANGES IN TOTAL CALCIUM CONTENT OF THE BONES DURING THE DEVELOPMENT OF RICKETS

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The metabolic disturbance leading to the development of rachitic bone changes is characterized by an impaired absorption of calcium and phosphorus from the intestinal tract. Whether the decreased absorption of calcium or that of phosphorus is of primary importance, and to what extent the one disturbance is dependent on or caused by the other, is still a moot question. The great difficulty of solving this problem by direct measurements of intake and output is vividly demonstrated in the recent papers of Nicolaysen ('37 a, b, c) and Innes and Nicolaysen ('37) where the question of absorption of exogenous calcium and phosphorus, of re-excretion into the intestines and further re-absorption is thoroughly discussed and further elucidated by ingenious experiments. This author concludes that, "in the rat . . . . the action of vitamin D in the gut is confined to a direct action on the absorption of calcium. The well-known reduced absorption of phosphorus in vitamin D deficiency is due to a precipitation by the increased amount of calcium in the bowel." This statement would reinstate calcium into the role of prime offender, a role that it has not played since the discovery of the low serum phosphorus in rickets and the low phosphorus diet for the production of experimental rickets.

Even though the direct evidence may, perhaps, still be open to discussion, there is indirect evidence indicating that the

metabolic disturbance in rickets may be accompanied by a calcium deficiency. The enlargement (Erdheim, '14; Ritter, '20; Pappenheimer and Minor, '21) and increased function (Hamilton and Schwartz, '33) of the parathyroid glands is one such piece of indirect evidence; the association of rickets and tetany is another. If, however, an insufficient absorption of calcium from the intestine is a regular feature of the metabolic disturbance of rickets, then it becomes rather surprising that this deficiency does not always lead to a decrease in the calcium concentration of the serum, just as the decreased phosphorus absorption always seems to result in a fall in the concentration of serum phosphorus. We must, then, assume that the concentration of calcium is maintained at a normal level by some special mechanism, perhaps, for instance, by the parathyroid hyperactivity just referred to. No amount of parathyroid hyperactivity could, however, supply the tissue fluids with sufficient calcium, if the absorption from the gut were considerably decreased. All that the parathyroids could do would be to govern, in some way, the mobilization and distribution of some endogenous store of calcium.

The only store of calcium in the body is contained in the bones. The mobilization of bone calcium can be studied only by determining the changes in the total amounts of calcium in the bones. A change in the total amounts of minerals in a bone will take place when there is a difference between the amounts deposited in new bone and in the amounts liberated by the resorption of old bone. This difference, the mineral balance of the bone, has not the same significance as decalcification or increased density of the bones. A negative balance must always lead to decalcification, but so may a positive balance, if it is too small in proportion to the rate of growth of the bones.

It would, a priori, seem highly probable that the calcium balance of the bones in rickets is a negative one. The histological examination shows, in severe cases, no evidence of calcification, while bone resorption seems to continue at a normal or even increased rate (Eliot and Park, '37). This histological picture would indicate that the total amounts of calcium

in the skeleton are decreasing, and the purpose of the present work was to check up on the impression thus obtained; to determine the changes in total calcium content of the bones during the development of rickets.

METHODS

To solve this problem, the only completely satisfactory procedure would be to use some method by which the total calcium could be accurately determined in the skeleton of a living animal, then produce rickets in that animal and, after a suitable period, repeat the determination. As no such method

TABLE 1

Comparison of metatarsals of both legs removed at the same time

NO.	DIET	TOTAL Ca MILLIGRAMS			LENGTH, MILLIMETERS		
		Left	Right	Difference	Left	Right	Difference
51	Normal	52.0	51.2	0.8	19.0	18.9	0.1
54	Normal	80.8	80.8	0.0	23.2	23.1	0.1
61	Normal	60.0	61.8	1.8	21.0	21.1	0.1
62	Normal	96.8	95.2	1.6	20.8	20.8	0.0
65	Normal	43.2	42.5	0.7			
67	Normal	47.2	44.4	2.8	18.1	18.0	0.1
68	Rachitogenic for 1 week	49.2	50.4	1.2	20.1	20.1	0.0
72	Rachitogenic for 1 week	110.0	110.0	0.0	24.4	24.2	0.2
73	Rachitogenic for 1 week	41.6	41.0	0.6	19.8	19.9	0.1
75	Rachitogenic for 1 week	47.8	46.8	1.0	19.4	19.6	0.2

exists, we have instead, compared the calcium content of corresponding bones. The amputation of one leg is easily performed and is generally well tolerated by young rabbits; after a period on rachitogenic diet the animals may be killed and the bones of the other leg analyzed. The diet used for the production of rickets was the McCollum diet 3143 (McCollum et al., '21) and the degree of rickets was estimated by x-rays taken just before the animals were killed. The bones analyzed were the metatarsals, and it may be seen in table 1 that the right and left metatarsals, when removed at the same time, contain about equal amounts of calcium, the greatest difference being somewhat less than 3 mg. The difference

obtained when measuring the length of the bones was never greater than 0.6 mm.[1] In order to determine the effect of the amputation on growth and calcification, normal animals on stock diet and of approximately the same age as the experimental animals were operated upon and killed at the same time intervals as the latter.

The bones, after being dissected free from adhering soft tissue were dried at 110°C. and then ashed at 400°C. Calcium was determined by the method of Fiske and Logan ('31), phosphorus by the method of Fiske and Subbarow ('25).

TABLE 2

Change in metatarsals in first 4 weeks of rachitogenic diet

NO.	DEGREE OF RICKETS	TOTAL Ca MILLIGRAMS			LENGTH, MILLIMETERS		
		Initial	Final	Difference	Initial	Final	Difference
445	+++	102.7	108.0	+ 5.3	23.9	27.8	3.9
408	+++	99.9	102.3	+ 2.4	24.5	26.9	2.4
155	+	60.4	62.6	+ 2.2	20.7	22.2	1.5
153	+	92.0	86.8	− 5.2	22.4	23.6	1.2
164	±	114.2	101.5	−12.7	24.7	25.1	0.4
434	++	159.2	143.6	−15.6	26.8	27.6	0.8
159	+++	146.1	121.8	−24.3	25.1	25.8	0.7
417	+++	181.6	148.6	−33.0	27.8	29.4	1.6
7	Control	64.0	114.4	+50.4	17.0	23.5	6.5
173	Control	126.2	153.4	+27.2	22.7	28.3	5.6
166	Control	92.9	115.6	+22.7	22.7	25.1	2.4
10	Control	76.8	97.3	+20.5	18.6	25.0	6.4
2	Control	65.6	79.6	+14.0	19.2	26.0	6.8

RESULTS

In the first group of animals one leg was amputated on the day that the rachitogenic diet was started, then, after 4 weeks, the animals were killed and the second leg removed. The controls were animals of approximately the same age, who were operated on and then killed at the same time intervals as the experimental animals, but fed on standard stock diet. Table 2 shows that in these normal animals the total amounts

[1] This difference was observed in a case in which the analysis was lost and that, therefore, is not included in the table.

of calcium in the metatarsals increased appreciably during this period of 4 weeks and that the bones also showed a considerable increase in length. Of the rachitic animals, three showed a very slight increase of total calcium (in two of these the increase falls within the limit of error of the method as shown in table 1). In the remaining five animals the total calcium decreased, in some quite markedly. There is a certain rough relationship between the behavior of the total calcium and the increase in length of the bones, the best increase in length being found where there was no loss of calcium.

TABLE 3

Change in metatarsals from fourth to seventh week of rachitogenic diet

NO.	DEGREE OF RICKETS	TOTAL Ca MILLIGRAMS			LENGTH, MILLIMETERS		
		Initial	Final	Difference	Initial	Final	Difference
422	++++	135.2	133.6	— 1.6	30.0	32.2	2.2
145	+++ (healing)	165.6	145.6	—20.0	32.0	34.1	2.1
149	+++	125.0	101.0	—24.0	24.8	25.7	0.9
421	++ (healing)	126.6	83.6	—43.0	25.0	26.2	1.2
90	Control	166.0	211.0	+45.0	32.2	34.1	1.9
139	Control	147.8	180.4	+32.6	26.0	28.3	2.3
137	Control	217.0	235.0	+18.0	32.0	34.2	2.2

In the second group (table 3) the experimental animals were given rachitogenic diet for 4 weeks before the amputation was performed. The diet was continued for 3 weeks after the operation, then the animals were killed and the second leg removed. The animals in this group did not stand the operation as well as those in the previous group, and those reported in the table were the few survivors of a much greater number of rabbits operated upon. The controls were animals of approximately the same age, fed on a normal stock diet, operated and killed at the same time intervals as the experimental animals. The controls showed an increase of the total calcium of about the same magnitude as found in the previous group. Of the rachitic animals, two showed in the x-ray definite signs

of healing. This somewhat complicates the interpretation of the results, as the experiment, probably, has included a period both of progression and regression of the rachitic process. The degree of this process, as marked in the table by plus signs, indicates the degree of rickets which seemed to have been present before the healing began. Both these animals showed a marked loss of calcium. In the two remaining, both with marked rickets, we find one with practically no loss of calcium and a growth in length as good as that of the controls, while the other has lost a considerable amount of calcium and grown very little.

In all cases we have also determined the amounts of total phosphorus, but as all our determinations were made on the whole bone, including the marrow, we have considered the figures obtained only as a rough check on the determinations of calcium, and have not included them in the tables. We will mention, however, that as soon as the change in total calcium was more than 3 mg., the total phosphorus always showed a change in the same direction, with the exception of case 164, where the phosphorus showed an increase of 3.6 mg., while the calcium decreased.

COMMENT

The rachitic animals and the controls behaved differently as far as the total calcium content of the metatarsal bones is concerned. All the controls showed a considerable increase, while the rachitic animals either lost calcium or showed a very small, practically negligible, increase. This difference cannot, of course, be due to the amputation, as all the animals were subjected to this operation. We do not think that it was due to any difference in activity, as none was noted, and the amount of activity of any rabbit in the ordinary laboratory cage is always very small. The diet must be responsible for the loss of total bone calcium and as we have previously shown (Hamilton, Kajdi and Meeker, '30) that on this diet the rabbit does not develop any acidosis, which might have led to decalcification, we feel that the loss of calcium is, in some way, directly related to the fact that the animals developed rickets.

We assume that this negative mineral balance of the bones is due to a lack of calcification, while bone resorption continued at a more or less normal rate.

As rachitic animals continue to grow, there must be an increase in the total amounts of phosphorus contained in the soft tissues. The characteristic features of the phosphorus metabolism of the body in rickets would be, then: the total amounts of phosphorus in the bones show a decrease; the total amounts of phosphorus in the soft tissues increase; the phosphorus concentration of the blood plasma and tissue fluids falls, presumably due to lowered absorption of phosphorus from the gut. We must conclude from these facts, that the soft tissues are able to utilize the phosphorus of the tissue fluids at a level of phosphorus concentration at which deposition of phosphorus cannot take place in the bones. The effect of this difference in efficiency between the soft tissues and the bones is that the phosphorus liberated from the latter by normal bone resorption is now available for the soft tissues. As soon as that level of plasma phosphorus is reached at which no more deposition of phosphorus can take place in the bones, a new supply of phosphorus is made available to the body.

As soon as the mineral balance of the bones becomes negative, not only phosphorus but also calcium is liberated. As the need of the soft tissues for calcium is very small, the circulating fluids will now have an abundant supply of calcium at their disposal. In some of our cases the amounts of calcium liberated from the metatarsals of one foot were about 1 mg. per day, and if the rest of the skeleton loses calcium at about the same rate, it would be a conservative estimate that some 10 to 15 mg. of calcium per day would enter the circulation. As the endogenous calcium losses of an adult man are estimated at less than 100 mg. per day (Bauer, Albright and Aub, '29), the negative calcium balance of the bones would certainly be sufficient to cover the losses by excretion of a rabbit weighing about 500 gm. Even if very little calcium were absorbed, the negative balance of the bones should be sufficient to maintain the calcium concentration of the body fluids at a normal level.

If, in rickets, the absorption of calcium is decreased, then we would expect more severe manifestations of calcium deficiency in mild than in severe rickets. In the latter, the bones would have a negative calcium balance and the requirements of the tissue fluids would thus be filled. In mild rickets, however, with a less severe impairment of the phosphorus absorption, the concentration of plasma phosphorus might, at times, increase to a value where calcification of bones could take place. As soon as that happened, the endogenous supply of calcium would decrease and any deficiency in the absorption of calcium from the intestines would be reflected in a decrease of the calcium concentration of the tissue fluids. This may be the reason why tetany is seen only in cases of mild or healing rickets.

It seems, then, that we do not have to assume the existence of any unknown mechanism in order to explain why, in rickets, the serum phosphorus is low while the serum calcium is normal. The only fact that may need further study is the ability of the tissues to utilize phosphorus from a lower concentration of plasma phosphorus than the bones. This tends to keep the tissue fluids permanently low in phosphorus, and as the low phosphorus makes calcification of bone impossible, all the calcium liberated by the absorption of old bone is now available to maintain the calcium concentration in the tissue fluids at a normal level. Our determinations show that the calcium thus set free ought to be quite sufficient for that purpose, even if the exogenous supply of calcium would be considerably diminished.

SUMMARY

The change in the total amounts of calcium in the metatarsal bones of rabbits was studied during the development of rickets. The method employed was to amputate one leg at the beginning of the experimental period and compare the calcium content of the bones of this leg with that of the other, removed after the end of the period. Suitable controls showed that the calcium content of the bones of one leg was very nearly the

same as that of the other when the legs were removed at the same time; and that in normal animals the amputation of one leg did not prevent the bones of the other leg from growing and increasing in total calcium content.

In seven out of twelve cases the calcium balance of the bones was found to be markedly negative; the amounts of calcium liberated from the metatarsal bones of one leg ranged in these seven cases from 0.45 to 2.10 mg. calcium per day. In the five remaining cases the balance was practically zero.

Even if the amounts of calcium absorbed from the intestines were considerably decreased, the amounts liberated from the bones would, in many cases, be sufficient to maintain a normal concentration of calcium in the tissue fluids.

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AN EASILY CONSTRUCTED RAT METABOLISM APPARATUS WHICH AUTOMATICALLY RECORDS OXYGEN CONSUMPTION AND ANIMAL ACTIVITY

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TWO FIGURES

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Uncontrollable activity of the common laboratory animals makes accurate determination of their true, basal respiratory metabolism by any of the usually employed methods almost prohibitively time-consuming; and may even make impossible the securing of necessary records at some critical period of an experimental procedure. This is not so much because they are never in a basal state as because there is no adequate, objective criterion by which it may be known when they are; nor methods sensitive enough to measure accurately such short basal intervals as frequently occur; by the time attainment of basal conditions seems a reasonable guess and an experimental run requiring considerable time for completion is gotten under way, the animal will have moved and it is necessary to begin anew. Nor are attempts to evaluate and thus salvage the less doubtful data by means of an accompanying activity record too helpful; for the usual mechanical registration of activity is, at best, only roughly qualitative; is influenced more by kind than intensity of movement; and there is often most unsatisfactory agreement between the apparent restlessness of the animal and the record of its respiratory metabolism.

In view of these generally conceded difficulties it is surprising that greater effort has not been made to develop methods of objective recording, sufficiently sensitive and accurate to make possible determinations of metabolic rate from basal periods of even short duration. A continuous graphic record of oxygen consumption, or carbon dioxide production, or both, of such sensitivity would, at the same time, automatically eliminate all doubt as to the occurrence or effect of activity; this would be immediately apparent and measurable in its only significant effect, viz., quantitative alteration of the respiratory exchange.

Efforts toward this end have been made; the first one on our part was to secure continuous, graphic records of the changes in weight involved in the use of Haldane's method, as was done independently by Arnoldi and described and used by Asada ('23); in our experience the rubber hose connections between the different parts of the apparatus so interfered with the free movement of the balances as to make the method useless. Next it was attempted to register graphically the movement of spirometers, for oxygen measurement (Hemmingsen, '34; Amano, '35); but our ingenuity was inadequate to devise any type of spirometer sufficiently responsive and sensitive to be of use with animals as small as the rat.

Attention was then turned to adaptation for this purpose of the 'automatic microspirometer' of Hanan ('29); in this ingenious device water from a constant-level reservoir siphons automatically into a burette as rapidly as oxygen is withdrawn from it by the animal; and in its original form it had been successfully applied to the rat metabolism apparatus of Foster and Sundstroem ('26) by J. O. Ralls of this school (unpublished). Although automatic in its operation, this device provided no continuous, graphic record of the volume changes, such as we wanted. Our first attempt to add this refinement was along the line later developed by Lewis and Luck ('33), whereby, upon reaching a certain volume, the displacement water automatically siphons out, record being made of the number of siphonings and thus of the oxygen used.

Though graphic, however, such a record is discontinuous; further, in our experience, the siphonings were never clean-cut and exact and no form of siphon we could devise would make them so. A still further objection to this method as thus employed is that the displacement water is continually renewed and, unless precaution were taken (as Lewis and Luck did) previously to saturate it with oxygen, would absorb indeterminate amounts of this gas as it was brought into contact with it (in the burette) in pure form.

The water displacement method, however, is very sensitive; it involves no mechanical moving parts with friction and inertia; all that is needed is a satisfactory method of recording the displaced volumes graphically and continuously; and, preferably, of operating it as a closed system whereby the same water might be used repeatedly and thus become and remain saturated with the gas (oxygen) with which it is in contact. With this experience and idea in mind the following apparatus was evolved, which among other advantages can be assembled almost entirely from odds and ends obtainable about any laboratory.

DESCRIPTION OF APPARATUS

Figure 1 is entirely diagrammatic; description and dimensions of the essential parts are as follows.

M^1–M^2 is a water-manometer system of which M^1 is a glass tube, 270 mm. long by 16 mm. diameter, carrying a paraffined, cork float; this float supports a writing point which traces a record (fig. 2) on smoked kymograph paper; the length of M^1, as given, was so chosen that the maximum excursion of this float and writing point would be from the bottom to top of our kymograph paper during a 30-minute run, i.e., provide a maximum vertical displacement and, hence, accuracy of measurement. M^1 and M^2 are connected with flexible rubber tubing to permit placing M^1 in relation to M^2 so the difference in water level will properly counterbalance the pressure (negative; see below) above the water in M^2. The connection of M^2 to the rest of the apparatus is also by flexible rubber tubing

(although not so indicated in the diagram) so that M^1–M^2 may be placed at any convenient position for securing the kymograph record, irrespective of the location of the rest of the

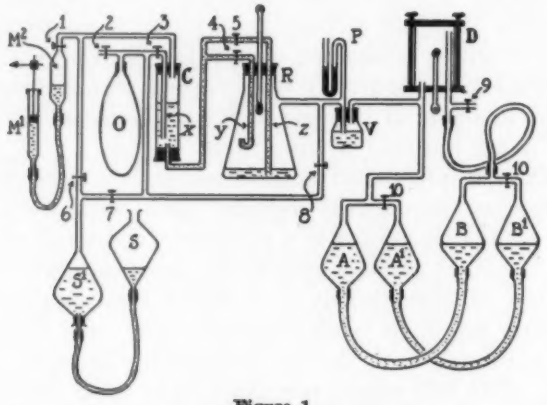

Figure 1

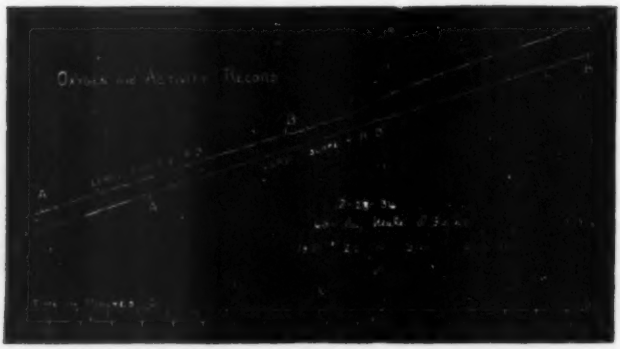

Figure 2

apparatus—as when the latter is in a constant temperature water bath or on another table, etc.

O is a rubber football bladder.

C is a glass cylinder 200 mm. long by 35 mm. diameter, provided at the upper end with a two-hole and below with a one-hole rubber stopper; these dimensions, again, were selected

to meet the particular requirements in mind and would necessarily be altered for use with larger animals, longer runs, etc.

The tube connecting C with R enters the latter by two arms; one of these, Z, extends to the bottom of R and is used (as described later) to draw the water back from R to C; the other, Y, shown with a bent tip, is of such a length that its open end in R is exactly at the same level as the lower end of the tube, X, which extends from the top nearly to the bottom of C; thus ensuring exact equality of pressure at the open ends of X and Y. Since, during operation, oxygen enters C through X from the bag, O, at atmospheric pressure (Mariotte burette principle), water will siphon from C to R through Y in proportionate amount only as the pressure in R tends to be reduced by withdrawal of its oxygen by the animal.

R is a 1-liter suction flask provided with a three-hole rubber stopper to carry a thermometer and the tubes Y and Z just referred to; R might be any size; the suction flask was the most convenient thing at hand for our use.

C and R together contain about 350 cc. of water to which, and to the water in the manometer system M^1–M^2, enough $CuSO_4$ is added to prevent mold growth. This amount of water in C–R is dependent on the size of animals used, length of run desired, etc.

P is an oil manometer for determining changes in pressure in the above system.

V is a 150 cc. wide mouth bottle or flask containing paraffin oil and inserted at this place as a Müller valve to prevent back diffusion and escape from absorption of carbon dioxide from animal chamber D.

D, the animal chamber, is a fruit jar, 190 mm. long by 80 mm. diameter, clamped between two heavy plates; the plate closing the open end of the jar (with a heavy rubber washer) is provided with brass tubes, threaded and soldered into it, for the necessary connections and insertion of a thermometer (in one model the thermometer was completely within the jar attached to the ventilation tube which extends to the bottom of it).

A–B, A′–B′ and S–S′ are leveling bulbs of 350 cc. capacity with openings at top and bottom of 22 to 25 and 3 to 4 mm., respectively.

A–B and A′–B′ are joined by their larger openings with 16 mm. rubber tubing; each pair contains 400 cc., 0.1 N barium hydroxide for absorption of carbon dioxide; for this purpose only one set, A–B or A′–B′, is used at a time by appropriate placement of clamps 10; circulation of air from A (or A′) through D to B (or B′) is obtained by raising and lowering B–B′, which are attached (by easily manipulated clips) to the arm of a motor driven crank, making about five revolutions per minute; this provides ventilation of the animal chamber at the rate of about 150 liters per hour.

S–S′ contains water.

A cylinder of commercial oxygen (not shown in the diagram) is attached beyond clamp 2 to replenish the supply in O when necessary. Also, before operation, C and R are flushed through and left filled with oxygen.

OPERATION

Oxygen determination

During preliminary periods of adjustment or at any other time when no record is wanted of the oxygen used, all clamps are tightened except 8; this permits oxygen to pass from the bag, O, through 8 and valve V into the animal chamber.

To secure record of the oxygen consumption: the apparatus has been previously set (as described below) so that 1) the water in the system M^1–M^2 is nearly all in M^2; thus, arm M^1 will be nearly empty and the float at its lowest point; 2) C is nearly full of water; 3) all clamps are tightened except 1, 3 and 4, which are loosened.

As the apparatus thus stands (with clamp 1 open) the manometer system M^1–M^2 will record the pressure above the water column in C; this will be negative (in millimeters of water) by an amount equal to the height of the water level in C above the opening of tube X; tube X connects (through 3, which is open) with oxygen at atmospheric pressure in O; the

open end of X in C is therefore the only point in C at atmospheric pressure.

As the animal uses oxygen, this must come (since clamp 8 is closed) from R; this tends to lower the pressure in R and water will siphon into R from C (clamp 4 is open, 5 is closed) through tube Y. Since, as explained before, the opening of tube Y is at the same level as that of tube X (in C) the pressure at the open end of Y will be the same as that at the open end of X, or atmospheric; thus the pressure of oxygen in R is atmospheric and as such, except for the very slight pressure required to operate valve V, reaches the animal.

As water siphons from C to R the level in C falls, decreasing the height of the column above the opening of X (atmospheric pressure) and thus decreasing the negativity of the pressure in the space above the water; therefore water will run from M^2 to M^1 and the float will rise, giving a record (on smoked kymograph paper) as in figure 2.

The advantage of this method of recording the displacement of water from C to R as a measure of oxygen used is that it permits the recording manometer system, M^1–M^2, to be placed at any convenient location irrespective of relation to the rest of the apparatus; thus, with C–R immersed in a water bath for temperature control direct measurement of the water siphoning from C or into R is very inconvenient; and even with C–R accessible, as when the entire apparatus is in air in a constant temperature room, this method of recording permits a flexibility of manipulation that is very desirable. The advantage of a completely closed system over any other we have seen for recording oxygen by water displacement is that the water (in C–R) can be used over and over again, repeatedly (as explained below); and being always in contact with oxygen there is no possibility of error in oxygen measurement due to absorption of indeterminate amounts by the water with which it is in contact.

Capacities and dimensions given are such that for rats of 130 to 230 gm. weight complete emptying of C requires at least ½ hour.

Although any description of the re-setting of the apparatus for another run will sound very cumbersome and time-consuming, actually it can be done with an interruption of only 30 seconds or less between successive periods.

Resetting the apparatus

At the end of an experimental period, as seen above, most of the water of M^1–M^2 will be in M^1; and of C–R in R.

Open clamp 8 so the animal may have uninterrupted supply of oxygen directly from bag, O, during the following manipulations.

Close clamps 3 and 4 and open clamp 5.

Place S lower than S′, thus establishing a negative pressure above the water in S′.

Open clamp 6; the negative pressure in S′ is thus transmitted in two directions: 1) to M^2, thus drawing water from M^1 back into M^2 and lowering the float of M^1 to its zero or starting position; 2) to C, thus drawing water back into C from R through tube Z; the water levels in M^2 and C are thus put in position to start a new experimental period. (In practice these two operations are controlled and executed independently by manipulation of clamps 1 and 5.)

Since clamp 8 is open during the above operation, oxygen can fill R from O as water is drawn from R into C; also after closing clamp 6 and opening 7 the oxygen drawn into S′ (from C) can be forced back into O (and thus saved) by raising S; clamp 7 is then again closed.

Analysis of the record

Graphic record from which oxygen consumption is measured is shown in figure 2 which illustrates parts of two, consecutive, 30-minute runs. The slope of the curves at any point is determined by the rate of oxygen consumption; basal intervals are clearly evident as those portions of the curves with least slope (sections A–B in both curves); activity on the part of the animal is instantly evident in increased rate of oxygen consumption and a steeper slope of the curve (as beyond B

in the upper curve) and is thus automatically registered as a part of the oxygen record. In any case, vertical differences in heights multiplied by the calibrated volumetric equivalent of the manometer gives the volume of oxygen consumed; reduction to standard conditions (0°C.–760 mm.) is effected in the usual way.

It is not unusual for a rat to be perfectly quiet throughout a 30-minute run and we have a great many such experimental periods; but the advantage of this type of record is that it permits the selection of basal intervals in a run which would otherwise be wasted or doubtful due to the indeterminate effect of an indeterminate amount of activity and is thus not only a great contribution to accuracy but also vastly conservative of the operator's time.

Determination of carbon dioxide

It is usual to use one set of absorbers, e.g., A–B, for preliminary and intermediate periods for which no record is required. At the beginning of an experimental period, noting the exact time, and as nearly coincident as possible with commencement of the oxygen record (as described above) clamps 10 are changed to the opposite arms of the T's on which they are placed so as to open for use the fresh absorbers, e.g., A′–B′. At the end of the experimental period, again noting the exact time, and as nearly as possible coincident with termination of the oxygen record, clamps 10 are replaced so as to put back into use A–B. A′–B′ are removed, set aside for analysis and replaced by a fresh set with which a new experimental period may be begun as soon as the oxygen recorder has been reset and started as described before. Or the preliminary absorbers may be replaced by a fresh set while the experimental period is in progress and a second period begun without any intermission.

Aliquot portions of the barium hydroxide solution are titrated with standardized 0.08 normal HCl using phenolphthalein as indicator, and checked to within 0.02 cc. Precautions are taken to guard against undue exposure of the barium

hydroxide solution while loading the absorbers, taking samples and titrating. It was the practice to allow the barium carbonate to settle out and use only the clear supernatant liquid for the titrations. Truog ('15), Bergman ('25) and Mack ('30) investigating the conventional method of shaking barium hydroxide solution during titration point out that the presence of barium carbonate does not affect the results. Following a similar procedure, samples of barium hydroxide containing barium carbonate were titrated and compared to supernatant samples and were found to check well. However, Martin and Green ('33) investigating this same problem show that carbon dioxide is lost in solutions of barium hydroxide containing barium carbonate, if these solutions are stirred with carbon dioxide-free air and titrated with a standard acid stronger than 0.07 normal. They believe this to be due to the liberation of carbon dioxide from the suspended barium carbonate by local concentrations of the acid. It was therefore deemed advisable to use only the supernatant barium hydroxide solution and thus guard against possible loss of carbon dioxide.

As thus used it is not possible to distinguish between the truly basal and other portions of a $\frac{1}{2}$-hour run with respect to carbon dioxide production. From the total carbon dioxide and total oxygen of such an experimental period we believe it is possible, however, to derive information of value respecting even the true, basal respiratory quotient. We have always discarded completely any periods in which there was considerable, persistent activity. So far we have analyzed over 400 experimental periods on more than 200 rats that were acceptable in this respect; segregating these into three groups which 1) were strictly basal throughout, 2) showed slight and 3) moderate activity, it has been found that the average respiratory quotient is exactly the same for each, i.e., the amount of activity which we believe is consistent with a basal oxygen determination by this method appears to have no significant effect on the respiratory quotient. This evidence will be published in full later; if true it means that not only the basal

oxygen consumption (which is determined directly) but also the basal carbon dioxide production and respiratory quotient may be determined from the data obtained by this method.

Checking the apparatus

A great amount of time was wasted in the effort to devise an alcohol burner that would simulate the rate of oxygen utilization of a rat. Recourse was finally had to withdrawal of oxygen and introduction of carbon dioxide by means of a burette attached beyond stopcock 9 (fig. 1) at rates comparable to those actually encountered in experimental use. The maximum error in single runs was, for oxygen less than, and for carbon dioxide slightly over 1%; the averages for a large number of such checks showed there was no systematic error in either measurement.

SUMMARY

An apparatus is described for determination of the respiratory metabolism of rats, the significant feature of which is a means of obtaining continuous graphic record of oxygen consumption of sufficient sensitivity to permit selection and accurate measurement of strictly basal intervals of even short duration. This is effected by a modification of Hanan's microspirometer which permits registration of the water siphoning into the oxygen reservoir to replace oxygen as it is used by the animal. Carbon dioxide is measured only for longer intervals of ½ hour or more by absorption and titration in barium hydroxide; the carbon dioxide absorbers serve also as the pumps to keep the air within the closed system in circulation.

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THE EFFECT OF PROLONGED EXPOSURE TO LOW TEMPERATURE ON THE BASAL METABOLISM OF THE RAT

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In connection with work being done in this laboratory on the effect of prolonged exposure to cold on development, estrous cycles and organ weights of the albino rat, the question arose as to what effect, if any, this, also, might have on basal metabolic rate. After the work was well under way it was found that Benedict and MacLeod ('29 b) had reported observations on a few rats kept for 24 hours at 21°C. which indicated that such exposure elevated the basal rate 4.5% above that to be expected if the animals had been previously maintained at 28°C. Also, Ishida ('30), investigating the influence of season, found the basal metabolic rate of the rat at thermal neutrality varied inversely with the previous environmental temperature. According to Hemmingsen ('34) it is important in the evaluation of such results to take into account alterations of body temperature; when this is corrected for, exposure to 20°C. or above for 18 to 24 hours previous to the metabolism measurement, has no appreciable effect on the basal rate. The recent work of Gelineo ('35) on the effect of exposure to high temperatures, while related to the problem in its broad aspects is not immediately relevant and need not be discussed.

PROCEDURE

Having no idea what length exposure might be required to effect maximum change in energy metabolism, it was arbitrarily decided to use periods of 15, 30 and 60 days, during which the experimental animals were kept for the major part of each day in a cold room at average temperatures for the various groups of 7.8 to 12.2°C. (table 1, column 3). Daily, from 8 A.M. to 4 P.M., they were removed to the warm animal room to dry out the cages and, more particularly, because of an early impression that while in the cold they did not eat or drink properly to maintain normal vigor and growth. This was due to preliminary observation that animals kept in the cold failed after a time to gain normally in weight; and it was assumed that if removed to normal temperature for part of the day this might be corrected. As will be shown (see Results: body weight) this was later proved incorrect, but only after so much data had been accumulated under this regime it was deemed best to complete the work as presented here without changing it.

As may be seen from table 1, the major part of the work was done with three groups of fifty female rats. Each group was subdivided into a cold-room unit of twenty-five (II B, III B, IV B) and twenty-five litter-mate controls which were kept in the constant temperature, warm animal room (II A, III A, IV A). In addition, a 60-day group of males (I A and B) was included for sex comparison; these were chosen from the same litters as the 60-day females.

All of the groups were started on the experiment at the same age (65 days) and therefore at nearly the same weight (average, 130 gm.; the 60-day males averaged 5 gm. heavier than their sisters). The metabolism of the 60-day groups was measured in the fall; of the 30- and 15-day groups in the winter. The diet for all was Purina Dog Chow, which we have used for several years and found adequate to maintain growth, vigor and fertility. The animals were kept in wire cages in groups of five. Estrous cycles were followed daily and recorded according to the method of Emery ('31). Careful

TABLE 1

SERIES (TWENTY-FIVE ANIMALS EACH)		1 Av. body weight	2 Av. rectal temp., °C.	3 Av. envir. temp., °C.	4 Av. chamber temp., °C.	5 R.Q.	6 cc. O_2/gm./hr.	7 % increase	8 Cal./sq.m./24 hrs.	9 % increase	10 Cal./100 gm./24 hrs.	11 % increase
I-A	60-day warm-room series (M)[1]	239	36.2	25.5	29.2	0.737	1.06	+15.0	975	+10.1	11.97	+14.8
I-B	60-day cold-room series (M)	220	36.7	7.8	29.1	0.750	1.22		1073		13.74	
II-A	60-day warm-room series (F)[2]	170	36.4[3]	25.5	29.0	0.758	1.06	+16.1	881	+12.4	12.15	+16.1
II-B	60-day cold-room series (F)	153	36.3[3]	12.2	29.0	0.766	1.23		990		14.10	
III-A	30-day warm-room series (F)	149	36.6	26.7	28.7	0.761	1.20	+15.0	949	+12.7	13.63	+15.0
III-B	30-day cold-room series (F)	143	37.7	9.5	28.8	0.761	1.38		1070		15.68	
IV-A	15-day warm-room series (F)	134	36.2	26.7	29.1	0.762	1.25	+11.2	969	+11.4	14.52	+11.1
IV-B	15-day cold-room series (F)	134	36.9	9.5	29.2	0.757	1.39		1079		16.13	

[1] (M), males. [2] (F), females. [3] Represents only a few animals.

record was kept of both warm-room and refrigerator temperatures (table 1, column 3). It may be added that the albino rats of our colony are a hardy, fertile, normal-growing, Wistar strain, inbred for many generations and quite free from internal and external parasitism and organic disease.

Metabolism technic. The animals were fasted 24 hours previous to metabolism determination and during the last several hours of this period were in the warm metabolism room, which was controlled to $\pm 0.5°C$. The animal chamber of the metabolism apparatus was maintained at the critical temperature of the rat, the average for all groups during the determination being 29.0°C. (table 1, column 4) and the range of averages, 28.7° to 29.2°C., which is in the middle of the critical temperature range of 28° to 30°C.

To induce quiescence, light was shone on the animal chamber; as Horst et al. ('34 b) have shown, this has no effect on basal metabolism; local heating from the lamp, and dead-air around the apparatus was eliminated by use of an electric fan.

Estimation of respiratory metabolism was made with the apparatus previously described (Schwabe and Griffith, '38). At least two 30-minute runs, and more if necessary for unimpeachable basal oxygen determination, were made with each animal. As was explained in connection with description of the apparatus the particular advantage of the continuous, graphic record of oxygen consumption provided by it, is that it makes possible accurate measurement of basal portions of a ½-hour record which otherwise would be dubious or useless due to sporadic activity. Consequently, as far as accuracy of basal oxygen determination is concerned, the 30-minute runs did not need to be, and the majority were not, strictly basal throughout.

This is important in connection with the determination of carbon dioxide production and necessitates explanation of the respiratory quotients. Carbon dioxide was determined only for the total, ½-hour run; this, together with the total oxygen for the same period (which was easily computed from the

graphic record) permits calculation of the respiratory quotient. This might be considered the basal value only for those metabolism periods that were strictly so throughout. Early in the work, however, comparison of two consecutive runs, one of which might have been basal and the other with some activity, or of two with different amounts of activity, showed close similarity of quotients. And when the work was completed the more than 400 ½-hour records were segregated into three classes: 1) those basal throughout, and those showing 2) slight and 3) moderate activity; with the result that the average respiratory quotients of the three groups were identical. This analysis will be published in full later; the result may be used here, however, as indicating that the slight activity during a 30-minute run compatible with a rigidly basal oxygen determination had little or no effect on the respiratory quotient.

Body temperature was measured immediately after the animals were removed from the metabolism chamber with a 1-minute clinical thermometer inserted 1½ inches into the rectum. The animals were then autopsied and body measurements and organ weights determined.

RESULTS

The significant data of the experiment are summarized in table 1; of these the basal metabolic rate interests us most, but before considering it, attention may be given to the effect of the cold exposure on weight, body temperature and respiratory quotient.

Body weight (column 1). Exposure for longer than 15 days inhibits gain in weight; comparing the cold- and warm-room series of each group, the retardation is, for the 60-day males, 9%; 60-day females, 11%; and 30-day females, 4%. This is in spite of the fact, as determined at intervals for limited groups, that the exposed animals ate approximately 30% more food per day than the controls. Since approximately 87% of their food intake was during the night while in the cold room (which compares favorably with the 84% nocturnal ingestion of the controls), and since 8 hours were available during the

day in the warm animal room to supplement any deficiency due to inhibition by the cold, it would appear that failure to gain weight was probably not the result of curtailment of opportunity to eat.

Rectal temperature (column 2). Excluding the 60-day females (II A and B), too few of whom were measured to give reliable averages, it may be seen that exposure to cold results in increased body temperature of 0.5°, 0.7° and 1.1°C., for the 60-day males, 15- and 30-day females, respectively.

Respiratory quotient (column 5). This appears to be unaffected; the average for all animals is 0.755.

Basal metabolic rate. In order to facilitate comparison with the data reported by others this has been given in the units most frequently used: oxygen per gram weight of rat per hour (column 6); calories per square meter per 24 hours (column 8); and calories per 100 gm. weight per 24 hours (column 10). Computation of body surface was by Diack's formula ('30), and of calories, by the usual method, based on the respiratory quotients of the ½-hour runs, as explained above.

Exposure to cold increases basal metabolic rate. The increase per unit of body weight is identical in terms either of oxygen consumption, as actually determined (column 7), or of the calculated heat production (column 11), with a maximum of 15 to 16%, which is attained sometime between the fifteenth and thirtieth day of exposure and is maintained without change at least as long as the sixtieth day.

Per unit of body surface the increase (column 9) is the same as per unit of weight up to the fifteenth day; thereafter, it is 2.3 and 3.7% less for the 30- and 60-day females, respectively; and approximately 4.7% less for the 60-day males. This is undoubtedly a result of the deleterious effect of cold exposure on gain in weight which was referred to earlier; and it is a nice problem, but one which it would be fruitless to pursue here, as to which method of expressing the results is physiologically preferable.

In any terms, this increased metabolic rate, as determined on this limited sample is statistically significant. In order to test this most severely, analysis has been made of the data per unit of surface, which, as just seen, gives the lesser differences. In this unit, the mean differences divided by their standard errors give values of 5.8, 5.1, 4.4 and 4.9 for the 15-, 30- and 60-day females, and 60-day males, respectively (table 2). Since a difference which is three or more times its standard error has validity, it may be concluded we are dealing

TABLE 2

Formulae used: $\frac{S.D.}{\sqrt{n}} = S.E.$ $\sqrt{(S.E._1)^2 + (S.E._2)^2} = S.E.$ of difference.

ANIMAL SERIES	RANGE, CAL./M.²/24 HRS.	MEAN ± S.E.	MEAN DIFF. / S.E. DIFF.
60-day warm-room (M)	820–1110	975±16	4.9
60-day cold-room (M)	930–1230	1074±17	
60-day warm-room (F)	720–1040	881±18	4.4
60-day cold-room (F)	760–1150	990±21	
30-day warm-room (F)	860–1130	949±13	5.1
30-day cold-room (F)	900–1300	1070±18	
15-day warm-room (F)	840–1130	969±14	5.8
15-day cold-room (F)	910–1170	1079±10	

here with a real effect. It may be important that the greatest significance, according to this test, attaches to the effect produced within the first 15 days. Here, again, the later results may be vitiated by the retarded gain in weight which is characteristic of the longer exposures.

DISCUSSION

Body temperature. The increased metabolic rate of the animals kept in the cold cannot be attributed solely to higher body temperatures since Hemmingsen's ('34) correction still leaves differences of 8 to 12% between the warm- and cold-room series of the various groups.

The average rectal temperature of both warm- (36.4°C.) and cold-room (37.1°C.) series is somewhat lower than that given in Donaldson's tables ('24) and reported by Benedict et al. ('32) and Horst et al. ('34 a). It has been pointed out that the rat is not strictly a homothermal animal; thus it is conceivable that the environmental temperature previous to the metabolism determination as well as the room temperature at which the body temperature is measured could appreciably affect the result; furthermore, the method of taking the temperature of such an animal as the rat is of importance, varies considerably and is not always stated. Because of these factors a discussion of the relation of body temperature to basal metabolism on the basis of this and other reported work seems inadvisable.

Age and sex. There is previous evidence (Hill and Hill, '13; Mitchell and Carman, '26; Houssay and Artundo, '29; Horst, Mendel and Benedict, '34 b, c; Landelius and Ljungkvist, '34; Davis and Hastings, '34) that the energy metabolism of the rat decreases with advancing age both per unit of weight and of surface and this is borne out by the data in columns 8 and 10 for both warm- and cold-room females; the slight difference between the 30- and 15-day groups is insignificant unless Hemmingsen's ('34) correction for body temperature is applied, when it, also, becomes appreciable.

Also in agreement with previous work (Mitchell and Carman, '26; Benedict and MacLeod, '29 b; and others), the metabolic rate of the 60-day males is approximately 10% greater than that of their litter-mate sisters per unit of body surface (column 8); this is true whether one compares the warm-room controls (I-A vs. II-A) or the exposed animals (I-B vs. II-B). On the other hand, per unit of body weight, the difference is negligible (I and II A, 1.5%; I and II B, 2.6%; column 10).

The very similar difference in weight between the warm- and cold-room males (I A and B, 19 gm.) and the warm- and cold-room females (II A and B, 17 gm.) together with the fact that the males were exposed to an environmental temperature

4.4°C. lower than the females, suggests that the male rat may be better able to withstand cold than the female. This might account for some of the apparent sex differences noted.

Amount of exposure. With the exception of the 15-day group, the rats exposed to low temperature did not gain in weight as rapidly as their litter-mates kept in a constant warm environment; exposure for longer than 15 days is deleterious.

Maximum increase in energy metabolism on the part of animals exposed to cold is attained by the thirtieth and sustained up to the sixtieth day. It would be of interest to know just when the maximum increase is reached; and, also, how much longer it would be maintained at this maximum level. Horst et al. ('34 c) have shown that slow-growing and stunted rats have a lower metabolic rate than rapidly-growing and normal-size animals. Since the cold-room animals do not maintain a normal growth but actually grow less and less rapidly as their stay in the cold is prolonged it will be of interest in future work to discover whether a time would be reached or body weight prevail when the enhanced rate declines; or whether the increased level will be maintained although the animals are progressively stunted in growth.

Respiratory quotient. The respiratory quotient of the fasting rat is at present such a controversial issue we do not expect acceptance of our figures as absolute, basal values without more support and defense than is in place here; this is planned for a later report. Our only concern here is whether the change in metabolic rate produced by cold was accompanied by qualitative changes in metabolism. And since all determinations were made under the same rigidly controlled conditions we can see no reason to doubt that significant differences in respiratory quotient would have been detected had there been any. We believe the evidence may be accepted as conclusive that exposure to cold had no effect on the nature of the foodstuffs metabolized by the fasting rat in spite of the considerable elevation of metabolic rate.

Surface area and body weight relationship. The use of the Diack ('30) formula for this investigation was largely a matter of personal preference; although, as Diack points out, it

gives results more nearly in line with those for other animals and man. Considering only the 30- and 15-day warm-room, female controls, their average caloric output is 959 Cal./m.^{2}/24 hours. This figure is in agreement with that of others (Benedict and MacLeod, '29 a, b; Verzar, '29; Hari, '24; Goto, '23) when the Rubner formula which they used is applied to our data. The heat production of the male controls is slightly higher than Benedict and MacLeod ('29 b) report for male rats.

Cause of the increased basal metabolic rate. The cause of this increase in basal metabolic rate as a result of exposure to low temperature is not clear. Krogh ('16) has suggested that the increased muscular activity of an animal exposed to cold may be a factor increasing muscle tonus at rest and so affect the basal rate; presumably such an effect might last for some time and thus be a factor in the increased rate which this work reveals.

The work of Hildebrandt ('21) indicates that it is not due to thyroid regulation, because when that gland is removed and the animal exposed to a cold environment during its metabolism measurement, or as Ring ('36) recently reported, previous to its basal determination, the same difference prevails. Leites ('35) speaks of an autoregulation of metabolism—metabolites that take part in the regulation of metabolism similarly to endocrine products; but this is too indefinite to demand attention here except to indicate the, as yet, unknown cause of the effect we have observed and measured.

SUMMARY

Exposure of the albino rat to low temperatures (7 to 12°C.) for the major portion of each day, for 15-, 30- or 60-day periods: 1) increases basal metabolic rate as measured at thermal neutrality, 11 to 16%; this increase, per unit of weight, reaches a maximum and sustained level between the fifteenth and thirtieth day; per unit of body surface the effect is slightly less, though still statistically significant; 2) increases body temperature; correction for this still leaves a

significant increase in metabolic rate; 3) retards gain in weight; 4) does not affect the respiratory quotient.

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